ANTI-IMPLANTATION EFFECT

OF

A CHINESE MEDICINAL PLANT IN ALBINO RAT

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TO ALL EXAMINERS AND GRADUATE SCHOOL OFFICE

Murraya panicualta is a plant under investigation in a World Health Organization sponsored research contract. We are not allowed to disclose any original results without prior World Health Organization clearance.

Any citation or public disclosure of these results may jeopardize our patent and forfeit further World Health Organization support.

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TABLE OF ABBREVIATIONS

MP	*Murraya paniculata*
MP-EtOH	*Murraya paniculata* ethanol extract
MP-aq	*Murraya paniculata* aqueous extract
MP-$CHCl_3$	*Murraya paniculata* chloroform extract
MP-BuOH	*Murraya paniculata* butanol extract
DWE	dry weight equivalent
PD_1	day 1 of pregnancy
PSP_1	day 1 of pseudopregnancy
RIA	radioimmunoassay
Δ^4P	progesterone
E_2	estradiol-17β
LH	luteinizing hormone
FSH	follicle stimulating hormone
hCG	human chorionic gonadotrophin
PG	prostaglandin
TE buffer	tris-EDTA buffer
i.g.	intragastrically

ABSTRACT

The effects of *Murraya paniculata* extracts on implantation were investigated in the rat. Oral administration of MP-EtOH in a minimum of 15 gm DWE was effective in suppressing pregnancy. The effective dosage of chloroform and butanol fractions were 20 and 60 gm DWE, respectively. The anti-implantation activity was most prominant when given on PD_{1-2} and is not active on PD_3 or thereafter. Ovum implantation was delayed for approximately 24 hrs. Like the effect of estrogen, the plant extract produced a sustained increase in uterine weight in intact or ovariectomized immature rats. It also stimulated vaginal cornification in both pseudopregnant and ovariectomized rats. MP acted as an estrogen agonist in producing long term retention of nuclear estrogen receptor, parallelled with a full replenishment of cytoplasmic receptor, which in turn stimulated uterine growth. Decidualization was suppressed by a minimum of 10 gm DWE of MP-EtOH. This inhibition was all or none in nature. Concentrations of E_2 in serum were significantly reduced in MP treated rats on PD_3 and PD_5. On the other hand, a significant increase in serum concentrations of Δ^4P were observed on PD_5. A single injection of E_2 at 1100 or 1600 hr on PD_2 or PD_4, failed to abolish the anti-implantation effects of MP. The plant materials exhibited no adverse effect on the development of the fetuses and normal mating and pregnancy occurred after the pseudopregnancy consequent after MP administration.

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1 INTRODUCTION

Much time and effort has been devoted to the search for anti-implantation agents with the idea that these might prove to be acceptable postcoital contraceptive substances in humans. Precoital techniques involve some waste of contraceptive effort, require premedication, often have unwanted side effects, may have anti-esthetic and anti-erotic aspects, and are not as yet infallible. Many studies have been concerned with the hormonal aspects of the pre-implantation peroid, others have concentrated on changes in cellular functions and relationships. Part of the direction behind this kind of research has been the idea that collection of sufficient information about the specific processes involved would allow the development of an agent which could block the implantation process specifically without producing a systemic effect.

Many of the chemical compounds which are effective as postcoital contraceptive agents are not in true sense anti-implantation agents, since the number of compounds which specifically inhibit implantation are very limited. Some compounds exert effects on this stage and also on other early preimplantation processes such as egg transport or egg development. In the present study, the term anti-implantation has been broadened to include antifertility effect between fertilization and implantation.

The folklore of many cultures includes descriptions of potions, usually of plant products, that can act as abortifacients. Although these have not been scientifically validated, reports appear persistently in antropological literature. *Murraya paniculata*, when used postcoitally, is effective in suppressing implantation. It may impair further development of the fertilized ova, either by a direct embryotoxic effect, or indirectly, by altering the rate of tubal transport, or render the uterine environment unfavourable for implantation. In the present study, special emphasis has been put on the elucidation of the mechanism involved. This work was extended to include the assays of estrogenicity, determination of steroid hormones levels, binding capacities of the cytoplasmic and nuclear estrogen receptors and the studies of the development of endometrial sensitivity, the effect on subsequent pregnancy, as well as the teratogenicity of the plant.

1.1 Endocrine control of ovum implantation

1.1.1 Role of progesterone

In a historical context the first hormone shown to be involved in implantation was progesterone. In the vast majority of species there appears to be an absolute requirement for progesterone, and removal of the source of the hormone results in failure to implant (Corner, 1928), although the blastocysts may remain viable for several days (Weitlauf & Greenwald, 1968).

1.1.2 Role of luteal phase estrogen

Krehbiel (1941) and Weichert (1942) found that implantation could be precipitated in lactating rats by the administration of a small dose of estradiol. This results suggest a role for estrogens in implantation. It was demonstrated that a condition of artifical delayed implantation could be induced in pregnant rats by ovariectomy before implantation, followed by administration of progesterone. If a small quantity of estrogen is given in addition to progesterone, then implantation occurs at the normal time (Cochrane & Meyer, 1957). These findings lead to the hypothesis that a surge of estrogen occurs on the third day of pregnancy and is responsible for implantation (Shelesnyak *et al.*, 1963). It is known that the uterus in rats is sensitized for implantation by a surge of estrogen secretion occurring on day 4 of pseudopregnancy (Shaikh & Abraham, 1969) or pregnancy (Yoshinaga *et al.*, 1969; Shaikh, 1971). In the mouse, estrogen peak was observed between 1000 and 1200 hr of day 4 and was significantly different from the samples taken at other times (McCormack & Greenwald, 1974). In rats, ovariectomy and progesterone replacement before this estrogen surge results in delayed implantation (Psychoyos, 1963; Zeilmaker, 1963).

1.1.3 Role of estrogen secreted before ovulation

It was reported that implantation could be obtained from the transfered blastocysts without priming the

ovariectomized recepients with estrogen before progesterone treatment (Humphrey, 1969). This indicated that priming is not essential for implantation in the mouse. However, the number of implantation sites was very small and evidence from experiments using the decidual cell reaction suggests that estrogen priming is neccessary to obtain a full reaction in the stroma, comparable to that which can be elicited in pseudopregnancy (Finn, 1966; Finn & Martin, 1970). Without priming, only a small response is obtained. It thus seems a reasonable assumption that the estrogen secreted during proestrus plays a small, if nonessential, part in inducing uterine sensitivity to implantation.

To summarize, it is established in the rat and mouse that the pattern of hormone secretion during early pregnancy brings the endometrium into a state of sensitivity which is necessary for the implantation of the ovum.

1.1.4 Role of the pituitary and hypothalamus

The secretion of the ovarian hormones is controlled by the pituitary hormones and which in turn are under the influence of the hypothalamus. The involvement of the brain has been shown by the destruction of certain areas of the hypothalamus and the systemic administration of neuroleptic drugs (Bindon, 1969). Ergot alkaloids and related chemicals also inhibit implantation by an effect on the hypothalamus (Mantle, 1969; Mantle & Finn, 1971). There seems

to be little doubt that prolactin is secreted in early pregnancy, with the major peak in the plasma between 40 - 60 hr after mating (Morishige *et al.*, 1973; Watson *et al.*, 1975). Ergot alkaloids are known to suppress prolactin secretion (Doehler & Wuttke, 1974) and injections of the hormone, or progesterone, will reverse the antifertility effect of the drugs (Mantle & Finn, 1971). There is evidence that luteinizing hormone (LH) is also involved in the control of progesterone secretion. Antisera to LH will prevent implantation in mice and the effect is reversed by injections of LH or progesterone (Madhawa Raj *et al.*, 1968, Loewit & Laurence, 1970). In the mouse, an increase in LH secretion occur with a peak on day 4 of pregnancy, but the level of follicle stimulating hormone (FSH) remain low before implantation (Murr *et al.*, 1974). An injection of LH will precipitate implantation in hypophysectomized rats maintained in delay with progesterone injections (McDonald *et al.*, 1967).

Corpora lutea of the estrus cycle in the rat are non-functional in the sense that they do not secrete enough progesterone to prepare the uterus for acceptance of fertilized ova. In pregnant rats, corpora lutea are made functional by the mating stimulus. Prolactin and LH are the chief components of the pituitary luteotrophic complex during the first half of gestation, causing the corpora

lutea to secrete progesterone (Smith *et al.*, 1975; Madhawa Raj & Moudal, 1970). If the corpora lutea of pregnancy are to maintain a relatively high secretion rate of progesterone, 20α hydroxysteroid dehydrogenase, a soluble enzyme in the corpora lutea responsible for the conversion of progesterone to 20α dihydroprogesterone, must be suppressed. Prolactin, which inhibits this enzyme, and LH which stimulates steroidogenesis, thus regulate the activity of the corpora lutea (Hashimoto & Wiest, 1969). LH was shown to deplete ovarian cholesterol stores and to stimulate progesterone and 20α dihydroprogesterone synthesis and release (Armstrong *et al.*, 1964; Behrman & Armstrong, 1969; Hilliard *et al.*, 1964). Prolactin alone is ineffective as a stimulus of steroid hormone synthesis (Huang & Pearlman, 1962; Marsh *et al.*, 1966). In the rabbit, prolactin was shown to increase the storage pool of cholesterol in the ovary (Hilliard *et al.*, 1968) and in the rat it was shown that prolactin prevent the conversion of progesterone to 20α dihydroprogesterone by inhibiting the 20α dihydroxysteroid dehydrogenase (Weist *et al.*, 1968). Prolactin, however, has a permissive action upon progesterone biosynthesis. A greater stimulatory effect of LH was observed in the presence of prolactin (Armstrong, 1969).

In the rat, however, FSH is required to induce implantation. The activity of FSH is mediated by the

ovarian follicles, possibly for the enhancement of estrogen secretion (Raud, 1974). This concepts fits well with recent reports that FSH stimulates estrogen synthesis in the ovarian culture and in the granulosa cell incubation system (Moon *et al.*, 1975).

1.2 Decidualization

The function of the uterus is to accomodate and to form a structural union with the developing young. In response to the presence of an embryo, the tissues of the uterus undergo considerable transformation into characteristic type of cell, the decidual cell. The presence of large quantities of glycogen and fat in decidual cells suggests that the decidua may have a nutritive function. Following activation and attachment, the embryo grows rapidly and a readily available source of carbohydrate might be advantageous. The blood vessels in the decidua are very well developed and in close contact with the decidua. Substances synthesized in decidual cells might pass into the sinusoids and be taken directly to the embryo (De Feo, 1967).

During most of the reproductive cycle in mammals, the endometrium is refractory to implantation. The uterus will permit the blastocyst to implant only for very brief peroids, during which it is said to be sensitive or receptive. It may be that implantation follows the sequence: estrogen priming, progestational changes, estrogen action,

sensitization, blastocyst or artificial trauma, transfer of information to stromal cells, stromal cell transformation (i.e. to decidual cell; Porter & Finn, 1977). The early stages of implantation and decidualization thus involve a complex interplay among the hormones of the ovary, the endometrium and the blastocyst, triggering of the uterus and attachment of the trophoblast to the uterine epithelium. It is clear that the ovarian hormones start the process going, but the seuqence of events and their control after that are very difficult to antangle.

1.2.1 Artificial decidual formation

It was discovered that it is possible to provoke the decidual transformation in the uterus of the non-pregnant animal by artificial stimulations (Leob, 1908). The discovery makes possible critical investigation of the physiological factors, permitting the uterus to respond to stimuli and undergo decidualization without the complications contributed by the presence of fertilized ova, at the same time permitting analysis of the separate role of the ovum and uterus (Finn, 1971). Artificial stimuli, both chemical and mechanical lesion, have been shown to be potent evokers of the decidual reaction. The rat endometrium can be induced to undergo decidualization if it is traumatised such as crushing at the appropriate time in the cycle (Selye & Mckeown, 1935). Various substances have been tested for the ability to initiate

a decidual cell response when injected into the uterus of a pseudopregnant animal. Of many substance tested in the rat by Finn & Keen (1963), only two classes of solution initiated a reaction, they are oils and sulphated polysaccharides (agar, carragenin and heparin). The injection of a small quantity of oil into the uterus of pseudopregnant rat provides a simple method for studying the effect of the drug to decidual stimuli. Because the oil is inert and unaffected by hormones, it can be assumed that it gives a constant nidatory stimulus to the uterus. The use of oil as a decidual stimulus has therefore some obvious advantages, although it must be admitted that its usefulness depends to some extent on the assumption that the stimulus given to the uterus by oil is similar to that provided by a blastocyst (Finn, 1965). This assumption cannot be proven at present as the nature of the stimulus in either case is unknown.

1.2.2 Role of estrogen in decidualization

An important feature of the action of estrogen in the preparation of the uterus for implantation or decidualization is the biphasic nature of the response. Sensitization occurs when small doses are given, but with larger doses inhibition occurs (Nutting & Meyer, 1963; Finn, 1966; Smith & Biggers, 1968). Thus, the decidual reaction can be prevented in pseudopregnant rats and mice by the administration of estrogens (Rothchild *et al.*, 1940; Rothchild & Meyer,

1942; Velardo & Hisaw, 1951). Similar, in pregnant rats and mice it is simple to interrupt pregnancy by treating the animals with estrogens just before implantation (Parkes *et al.*, 1938; Dreisbach, 1959).

1.3 Estrogen receptor binding and relationship to uterine growth

It is well established that estrogen stimulate cellular hypertrophy and uterine hyperplasia, thereby increasing uterine weight. Once inside the cells of the target organ, estrogens act by influencing the transcription of information in the nucleus with the formation of new RNA, which then induces enzyme synthesis in the cytoplasm (Hamilton, 1968) and initiate morphological changes in the endometrium.

The initial events accompanying estrogen uptake by cells of estrogen-responsive tissues had been studied extensively (Gorski *et al.*, 1968; Jensen & DeSombre, 1972; O'Malley & Means, 1974). The current model for estrogen in target tissues indicates that after entering a target cell, the hormone binds to high affinity cytoplasmic receptor to form a complex which then enters the nucleus. Considerable evidence suggests that the presence of the receptor-estrogen complex in the nucleus is a prerequistite for stimulation of many estrogen-induced biosynthetic events (Katzenellenbogen & Gorski, 1972; Clark *et al.*, 1973; Ruh *et al.*, 1973). However, the nature of the events that are stimulated by an estrogen

in the immature rat uterus depends not only on the quantity of nuclear receptor complex but also on its duration in the nuclear compartment (Anderson *et al.*, 1975).

Maximal uterine growth correlates with the number of nuclear receptor-estrogen complex that undergo long term retention. This long term nuclear retention of the receptor-estrogen complex will initiate a series of early and late uterine responses. Early responses include water imbibition, hyperemia, amino acid and nucleotide uptake, activation of RNA polymerase I and II and stimulation of protein synthesis, whereas late responses include cellular hypertrophy and hyperplasia, sustained RNA polymerase I and II activity (Clark *et al.*, 1973; 1974; Rochefort *et al.*, 1972).

The retention of the nuclear receptor-estrogen complex is accompanied by a rapid replenishment (possibly via recycling and resynthesis) of the cytoplasmic receptor within 24 hrs. This replenished cytoplasmic receptor is then free to interact with estrogen to form receptor-hormone complexes which bind to nuclear sites and cause a second cycle of uterine growth stimulation (Katzenellenbogen & Ferguson, 1975).

1.4 Agents with anti-implantation effects

The successful initiation of blastocyst attachment is dependent upon the precise synchronization of endometrial, embryonic and ovarian function during the first

few days of pregnancy. The finely balanced nature of the mechanisms controlling implantation render this stage of development particularly susceptible to contraceptive attack. Several possible routes have been suggested:

1.4.1 Compounds affecting egg transport

The egg transport phase in the oviduct of most species occupies the first three or four days after ovulation (Croxatto *et al.*, 1972; Harper *et al.*, 1960). During the first day, while under estrogen domination, the eggs move quickly through the thin walled ampulla. Under a delicate balance of endogenous estrogen and progesterone, the eggs are held at the ampullary-isthmic junction until fertilized and then descend through the muscular ampulla to enter the uterus via the uterotubal junction. The eggs then move freely in the uterus until attachment (Bennett, 1975). Steroidal estrogens (Chang & Harper, 1966), and antiestrogens (Spona *et al.*, 1972), gonadotrophins (Harrington, 1965), prostaglandins (Ellinger & Kirton, 1972), autonomic drugs (Pauerstein *et al.*, 1973; Bennett & Kendle, 1967) and a large number of non-steroids act to accelerate or retard the rate of egg transport.

Ovarian steroid hormones have a major regulatory influence in the transport and normal development of zygotes in the oviduct. Estrogens increase the rate of secretion of tubal fluid (Mastroianni *et al.*, 1961),

are prerequisites for ciliary growth and movement at the ostial portion of the tube, and tend to increase the frequency and decrease the amplitude of tubal muscle activity (Hafez, 1975). Progesterone, in the presence of estrogen, tends to decrease the frequency of contraction and increase the amplitude. The overall effect of the ovarian steroids is to alter the responsiveness of the tubal musculature to local release of adrenergic agents (norepinephrine, Coutinho *et al.*, 1970). Upsetting the proper sequence in the hormonal environment can therefore alter moltility and disturb the normal passage of cleaving ova in the oviducts (Segal & Nelson, 1958). This has been demonstrated by many experiments, but species differences and variations in experimental techniques have made it difficult to construct a unified concept form reported observations.

In the view of the universality of estrogen effects on the oviduct and egg transport, it is perhaps not suprising that estrogens and antiestrogens are equally effective. In mice, the predominant anti-fertility effects of antiestrogens are to cause tubal retention of eggs. It was discovered that antiestrogens such as DES, MER-25, MRL-37, when given on days 1 to 3 of pregnancy, caused both retard transport and delayed development of eggs (Humphrey & Martin, 1968). Since all

these compounds exert some estrogenic activity (Emmens, 1965a; 1965b; Emmens & Martin, 1965; Greenwald, 1965), their effects on egg transport are due to its estrogenic property and not to their antiestrogenic properties.

A general held theroy is that changes in the rate of egg transport produced by a drug-induced alternation of endogenous estrogen level, producing a desynchronization of the biochemical and transport relationship between the egg and the reproductive tract, with consequent degeneration of the egg.

1.4.2 Compounds affecting egg development

It was generally concluded that most of the compounds which exert a direct effect on the developing egg are those which are highly toxic for all rapidly dividing cells (Harper, 1968b). Some compounds developed for cancer chemotherapy have been tested in pregnant animals. It is not suprising that oncolytic agents are able to interfere with the growth of embryonic as well as cancer cells. Methotrexate, 6-mercaptopurine are oncolytic drugs that produce remissions of trophoblastic tumors. Many compounds that act as mitotic poisons, inhibitors of protein synthesis, or antimetabolites will cause resorption or abortion of implanted embryos in experimental animals (Thiersch, 1962).

1.4.3 Compounds affecting function of the corpus luteum

In all species studied, a successful intrauterine pregnancy requires sufficient progestational support of the endometrium, both for preparation for nidation and for maintainance of the placenta. Early pregnancy requires adequate corpus luteum function. Afterward placental production of progesterone is capable of maintaining pregnancy in the absence of the ovaries. Several classes of compounds have been studied for their ability to suppress luteal phase progesterone, three approaches have been suggested:

1.4.3.1 The disruption of luteal function by inhibiting the early luteotrophic activity of the blastocyst

In women, the early conceptus exerts a direct luteotrophic factor, human chorionic gonadotrophin (hCG), which starts to rise about one day after implantation (Jaffe *et al.*, 1969; Saxena *et al.*, 1974) and quickly stimulates a rise in the plasma progesterone levels (Hanson *et al.*, 1971; Niswender *et al.*, 1972). By blocking the action of such factor, it should be possible to arrest pregnancy of the attachment stage. In order to acheive this objective, immunogens are being developed for the active immunization of women aganist chorionic gonadotrophin (Bahl *et al.*, 1976). The key to a possible immunological approach to contragestat-

ional activity is the selection of a specific antigen that will not cross react dangerously with other tissue antigens and that will selectively interfere with a critical event in the reproductive sequence. Another approach involves the development of antihormones to compete with hCG for receptor sites on the corpus luteum. These compounds might be administered in the event of a missed menses, or on a once-a-cycle basis at the expected time of menstruation, to induce luteal regression.

1.4.3.2 The disruption of luteal function by interfering with progesterone receptors in the endometrium

High affinity receptors for progesterone have been identified in the uterine cytosol of the calf, guinea pig, human and rat (Thomas, 1973; Milgrom *et al.*, Milgrom & Baulieu, 1970). The manipulation of receptor function offers great promise for the development of new contraceptive agents. Antiprogestins, such as 16α-bromo-acetoxy-progesterone and R-2323 are currently being sought which posscess the ability to compete with endogenous progesterone for uterine binding sites, but lacks progestational activity. These compounds could be used on a once-a-cycle basis to saturate the large number of free receptor sites present in the endometrium during the late proliferative phase.

1.4.3.3 The disruption of luteal function with prostaglandins or steroids which act directly on the corpus luteum

The mechanism by which $PGF_{2\alpha}$ induces luteal regression is poorly understood. In rats and mice, an important step in PG induced luteolysis appears to be the activation of an enzyme, 20α hydroxysteroid dehydrogenase, which catalyses the conversion of progesterone to a biologically less active derivative, 20α dihydroprogesterone (Fuch & Mok, 1974).

Estrogenic compounds have been found to induce luteolysis in laboratory animals (Guinea pig, hamster, rabbit and rat, Oriol-Bosch & Cortes, 1975). Systemically administered estrogens are also known to exert a powerful antifertility effect. There is some evidence to support a luteolytic mode of action (Gore *et al.*, 1973).

1.4.4 Natural products

Abortifacient substances have been found in various plants, but frequently the abortifacient activity can be ascribed to estrogenicity. This is probably the explantation of the activity of pine needle (*Pinus sylverstris*) extracts and subterranean clover (*Trifolim subterraneum*) in causing fetal resorption in animals. Mexican women are believed to use a plant called Zoapatle (*Montanoa tuberosa*) to induce abortion (Segal, 1977). Here

again estrogenicity may be involved, but a compound with oxytocic properties may also be present.

1.5 *Murraya paniculata*

1.5.1 Botany

Murraya, belongs to the Rutaceae, is frequently used in landscape gardening for its dark, glossy, green foliage, white fragrant flowers and rich-red fruits. Depending on the taxonomic opinions of various taxonomists, there are about six to sixteen species in the genum *Murraya*. The differences in opinions are mainly due to the wide range of variation in morphology among many species, compounded with the artificial selection of horticultural strains. Six species are found in China, namely: *M. alata, M. euchrestifolia, M. keonigii, M. microphylla, M. paniculata (M. exotica)*, and *M. tetramera*. *M. euchrestifolia* is endemic to Taiwan, while *M.tetramera* is found only in Yunnan.

Distribution of the *Murraya* species has a distribution center in southern China and the several countries connected to the southwest border of China, and spreads to India, Sri Lanka in the west, and Phillipines and Indonesia in the east.

A simple botanical description of *M. paniculata* Linn. Jack (syn. *M. exotica*) reads as follows: an evergreen shrub or small tree with very hard wood, sometimes attaining 15 ft in height. Glabrous with the

young parts soft-hairy. Leaves lustrous green, 4-6 inches long, with 3-9 leaflets, rarely reduces to one; leaflets oval-shaped, shortly pointed, 3/4 to 1/2 inches long. Flowers white, fragrant formed in tight terminal clusters during the summer. Although each individual flower is less than 1/2 inch in daimeter, they create a mass effect because of their abundance. Fruit, round, one to two seeded berry, bright-red, 3/4 inch in diameter.

1.5.2 Phytochemistry

Pharmacognostical work on *M.* species have been carried out since 1965. Twenty alkaloids were isolated from three *M.* species, including *M. paniculata* (Mester, 1973; 1977). By the end of 1979, at least six more alkaloids have been identified (Chakraborty *et al.*, 1978; Fauvel *et al.*, 1978; Ganguly & Sarkar, 1970). In 1978, Gray and Waterman (1978) reported that twelve coumarins have been found in different parts of *M. paniculata*. In addition, two coumarins, viz., auraptene and imperatorin were missed in this list (Ganguly *et al.*, 1977; Sanyal *et al.*, 1975). Up to now, chemical works were mainly concentrated on the aerial parts, i.e., leaf, flower, fruit and stem. Only one report concerning the chemical composition of the root of *M. paniculata* was found (Fauvel *et al.*, 1978).

1.5.3 Ethnomedical information

In traditional Chinese medicine, *M. paniculata* was used for the asperation of vital energy (行氣), invigoration of the circulation (活血) and the dispersion of the extravasated blood (化瘀) (Anon, 1977). In Singapore and Java, the aqueous decoction of the leaves of *M. paniculata* is used to cure abnormalities in female reproductive functions (Kan, 1972). It can also be used as emmenogogue (Cheung, 1970). The application of *M. paniculata* for the treatment of diarrhoea, dysentary, dropsy was reported by Chopra *et al.* (1956). *M. koenigii* Spreng, a related species, is used in Indian folklore medicine as an antiperoidic (Gupta & Nigam, 1971).

1.5.4 Pharmacology and clinical reports

A colourless crystalline non-nitrogenous compound was isolated from the petroleum ether (60°-80°C) extract of the leaf of *M. paniculata*. This compound has a relaxation effect on rat ileum, antagonize the histamine induced contraction but not the acetylcholine induced response. No significant effect on blood pressure or respiration was observed on anaesthetized dog (Anon, 1970). 50% ethanol extract of *M. paniculata* has been used as local anaesthetics for gastrectomy (Anon, 1970) and tonsillectomy (Anon, 1971).

Clinical use of the aqueous extract of

the root of *M. paniculata* for ecobolic has been reported by the Chinese scientists. The drug was applied intracervically to 177 patients, and 173 were reported to be effective (Anon, 1973).

2 EXPERIMENTAL

2.1 Effects of MP extracts on implantation

2.1.1 Animals

Sprague-Dawley Rats strain, originated from Charles River, were raised in Science Center, The Chinese University of Hong Kong. They were housed in animal quarters with artificial illumination from 0900 to 2100 hr daily. Vaginal smears were examined daily. Proestrus females were caged overnight with proven males. The appearance of spermatozoa in the vaginal smear in the following morning was taken as the first day of pregnancy (PD_1). Animals received graded doses of MP extract on different days of pregnancy. Doses were administered intragastrically (ig) in a maximum volume of 1 ml/day The extract were dissolved in vehicle (ethanol/Tween 80/ water : 2/1/7 v/v). Each dose was quantitated as the extract prepared from unit dry weight of MP. Control groups either received similar volume (1 ml) of the appropiate vehicle or not treated at all. All animals were autopsied at 1200 hr on PD_8. The number of live implantation sites were recorded. The length of the long axis of the implantation site was measured using a calibrator. Any animal having at least one or more implanted embryos was considered as pregnant. All animals, before and after mating were maintained on uniform husbandry

conditions. They were fed on a standard Purina laboratory diet with drinking water *ad libitum*.

The biological activity of the alcohol extract was tested in the Worcester Foundation for Experimental Biology, Charles River CD rats were used. These pregnant females were subjected to similar treatment schedule. Autopsy was performed on PD_8.

2.1.2 MP extracts preparation

The plant materials were obtained from various sources, some were cultivated in the University campus, some were purchased from Chiangmai or China. The air-dried root was washed in tap water to get rid of soil. It was cut into small chips with a garden shear and grounded into a coarse powder. The root was percolated with distilled water or refluxed in 70% ethanol. Detailed extraction and purification procedures were outlined in figure 1. Only the root of MP was used in this work. The quantity of various extracts were referred to dry weight equivalent (extracted) from unit weight of starting material. The origin of each batch of extract varied but the effective dose is fairly constant at 20-30 gm/PD_{1-4}. The alcohol extract of MP root was disignated MP-EtOH, the same applies to MP-$CHCl_3$ (chloroform) etc.

2.1.3 Delayed implantation

The onset of implantation was determined by the

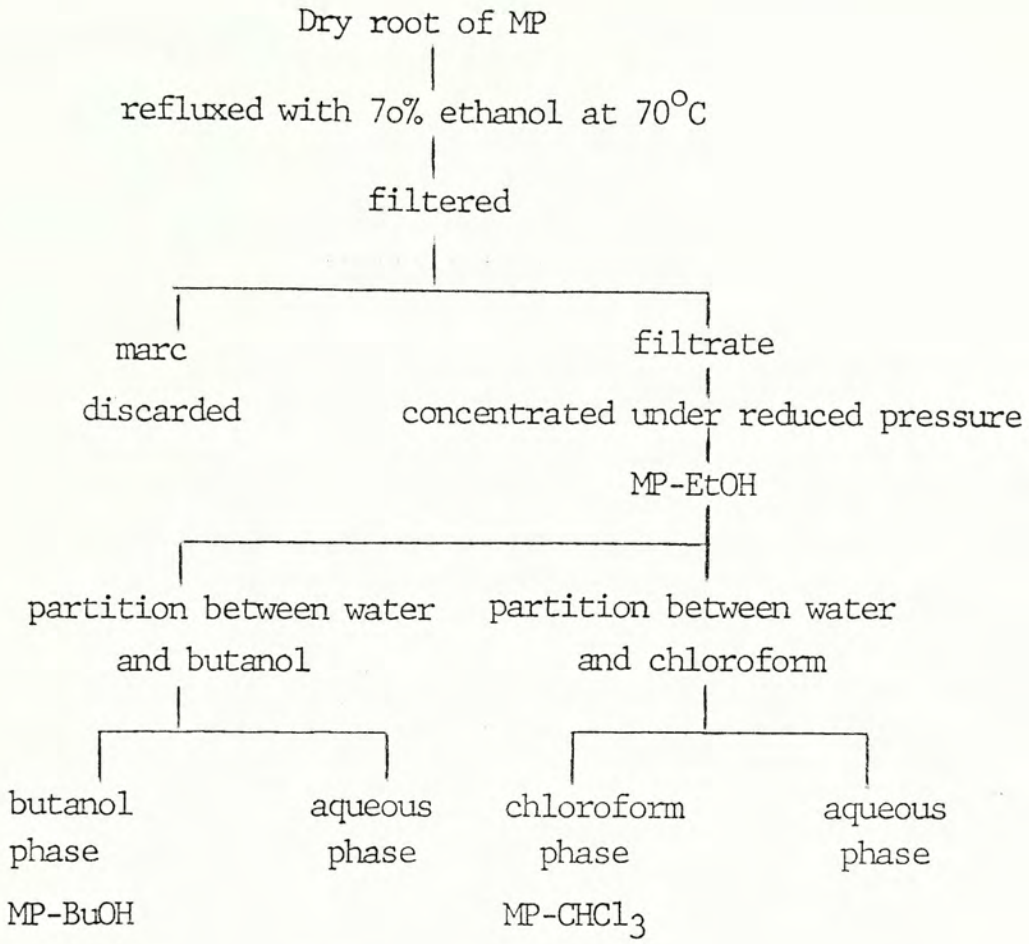

Fig.1 Outline of the extraction procedure of MP

i.v. injection of 0.2 ml 3% pontamine sky blue 6BX (Hopkin & Williams) in 0.9% (w/v) NaCl solution 10 to 15 minutes before sacrifice the animal (Psychoyos, 1971).

Animals were treated with MP-EtOH on the morning of PD_1 and PD_2 (5 gm DWE daily). Control animals received the vehicle alone. Groups of treated and control animals were killed at different time points on PD_5 or PD_6.

2.2 Assays of estrogenic activity

2.2.1 Vaginal cornification

Adult virgin rats, ovariectomized about 14 days before the experiment, were dosed daily for 6 days (day 1 to 6). Vaginal smears were taken each morning from days 1 to 7 inclusive, and also in the late afternoon (1600 hr) of day 4. Smears were classified as cornified which were composed of cornified and/or nucleated epithelial cells with no more than the occasional leukocyte. Rats which had a cornified smear at any time during the last 3 days of test were considered to have positive response.

2.2.2 Uterotrophic response

Immature female rats of 21 to 23 days old were used. Each test group comprised 5 animals. Ovaries were removed on day 1 of experiment and drug administration commenced on the same day. Group 1 received 1 ml vehicle daily (Ethanol/Tween 80/water : 2/1/7). In group 2, 2.5 µg/day estradiol-17β was given subcutaneously whereas in

group 3, MP extract was given at a dose level of 1 gm DWE/ day. Treatment continued once daily for 3 days and on the fourth day the animals were killed and their uteri excised, blotted and weighed.

The increase in mean uterine weight per animal was used as an index of uterotrophic effect.

2.2.3 Labeled estradiol exchange assay: Actions of MP-EtOH on the levels of uterine estrogen receptor and uterine growth

Estrogen receptor in the cytoplasmic and nuclear compartments were determined by the labeled estradiol exchange assay (Katzenellenbogen *et al.*, 1973; Anderson *et al.*, 1972) with slight modification (Katzenellenbogen, 1975).

Immature female rats of 21-23 days old were grouped and treated according to the following experimental design. Rats were injected subcutaneously with saline (control), or 2.5 µg estradiol (E_2), or 2 gm DWE MP-EtOH extract intragastrically (MP) or a combination of estradiol and MP-EtOH (MP+E_2). Two groups received one dose were sacrified respectively at 24 hrs or 48 hrs later. A third group received two doses 24 hrs apart were sacrified at 24 hrs after the second dose.

Animals were killed by cervical dislocation, uteri were rapidly removed, trimmed, weighed and placed in ice-cold Tris-EDTA buffer (10mM Tris-HCl, 1.5 mM EDTA, pH 7.4,

TE buffer). Uteri (3 per group) were rinsed with 20 ml TE buffer and then homogenized with a Polytron (Kinematica PCU-2) by three 5-sec bursts. The homogenate was centrifuged at 800 x g for 20 min to yield a nuclear pellet and a low speed supernatant. The nuclear pellet was washed 3 times, each with 2 ml cold TE buffer and followed by centrifugation at 800 x g for 10 min. The washed nuclear pellet was then resuspended in 13 ml TE buffer. The low speed supernatant was spun at 100,000 x g for 60 min in a refrigirated Beckman L3 ultra-centrifuge with type 40 rotor to yield the cytosol fraction which was further diluted to 13 ml.

0.8 ml of the nuclear fraction or cytosol fraction were added to tubes containing 5×10^{-10}M to 1×10^{-8}M labeled estradiol-17β. The incubation volume was 1.0 ml. The nuclear fraction was incubated at 37°C for 30 min. After the incubation period, tubes were placed in ice bath for 10 min and 1 ml ice-cold TE buffer was then added. The tubes were vortexed and spun at 800 x g for 20 min at 4°C. The supernatant was discarded and the pellet was washed 2 times with 3 ml ice TE buffer, each time was followed by centrifugation at 800 x g for 10 min. The final pellet was extracted with 2 ml ethanol. The ethanol extract was counted in a Beckman LS-330 liquid scintillation counter. The amount of radioactivity extracted by alcohol indicated the capacity of the nuclear receptor.

The cytosol fraction was incubated at 4°C for 18 hrs followed by the addition of 0.5 ml charcoal-dextran (10% v/v). The mixture was placed in ice for 10 min and then centrifuged at 2000 x g for 10 min. The supernatant was counted.

2.3 Actions of MP on decidual formation

Pseudopregnancy (PSP) was induced by mechanical stimulation of the cervix in the evening of proestrus and the morning of estrus (De Feo, 1963a; 1963b). The day of vaginal cornification was designated as day 0 of PSP (PSP_0). PSP_1 was characterized by the first marked appearance of leukocytes in the morning lavage. The stimulus for deciduoma initiation was provided on PSP_4 by 0.05 ml arachis oil injected into the lumen of the uterus through the top of the left uterine horn (Finn & Keen, 1963). The effect of MP on the decidual development was determined in hooded rats administered a total dosage of 10 to 40 gm DWE of MP-EtOH from PSP_0 to PSP_3.

Unilaterally pregnant rats were prepared by ligating the left follopian tube on PD_2. The left uterine cornu was injected with 0.05 ml arachis oil on PD_5.

The animals were anesthetized on the fifth day after the injection of oil; the uteri were removed and split at the bifurcation point; the mesentery was trimmed off, and each cornu was weighed, cervix included, on a

E. Mettler analytical balance. Rats with swellings in the left horn were considered to have positive response and the difference in weight between the two horns was taken as an estimate of the magnitude of the response.

2.4 Determination of serum steroids levels by radio-immunoassay

Blood samples (2 ml) were taken from the tail artery under ether anaesthesia during 0900 to 1000 hr on PD_3 to PD_5. No rats were bled more than 2 times. The blood samples were left to clot at $4^{o}C$ and 0.7 to 1 ml serum aliquots were then stored in glass scintillation vials at $-20^{o}C$ for deterimination of progesterone (Δ^4P) and estradiol-17β (E_2).

Measurement of Δ^4P and E_2 in serum was carried out after extracting once with 10 ml anhydrous ethyl ether. Tritiated steroids (2,500 dpm for each steroid) were added for the determination of recovery rate. The dried extract was dissolved in 1.0 ml of iso-octane and steroids were separated on micro-celite columns (Saksena *et al.*, 1977). The concentration of the steroids was determined by radioimmunoassay (Saksena *et al.*, 1976). The specifications of the antiserum (anti-estrogen, gift from Dr. B.V.Caldwell, and anti-progesterone, provides by Dr. G.E.Abraham) used have been described earlier (Saksena *et al.*, 1978).

2.5 Effects of exogenous estrogen on MP treated rat

Experiment was carried out to determine the effect of estradiol-17β, when supplement added, to prevent the delay in ovum implantation.

A group of 6 rats were treated with MP-EtOH as described in Expt 1. A single subcutaneous injection of 2.5 or 0.2 μg E_2, dissolved in 0.2 ml arachis oil, was given to these rats at 1100 or 1600 hr on PD_2 or PD_4. All rats were autopsied on PD_8 to examine for implantation sites.

2.6 Study of the teratogenic effects of MP

2.6.1 Effects of MP on sex ratio of the fetuses

MP-EtOH extract was administered on PD_1 and PD_2 in a total dosage of 5.0 gm DWE. Fetuses were examined on any morphological abnormality or sex reversal presents on day 3 postpartum. As the anogenital distance of the male fetuses is greater than that of female fetuses (Revesz *et al.*, 1960), this serves a good criteria in distinguishing both sexes.

2.6.2 Effects of MP on subsequent pregnancy

Animals were treated with two oral doses of MP-EtOH extract (10 gm DWE/day) on the morning of PD_1 and PD_2. Rats that were found not pregnant on the day of laporotomy (8 days postcoitum) were resutured and cared for. Upon the reappearance of a proestrus smear, they were

caged with males of proven fertility. It has been shown that a normal estrus cycle had returned and that mating had occurred. The numbers and sizes of the implantation sites in the experimental series were compared with those of the controls according to the Student t test.

3 RESULTS

3.1 Anti-implantation effects of MP extracts on pregnant rats

3.1.1 Control group

Normally in control group, the average number of viable implantation sites was 11.4, with a mean length of 0.46 cm. The rats treated with vehicle from PD_1 to PD_4 were pregnant and unaffected on PD_8, showing the number of a mean of 12.5 implantation sites and 0.44 cm mean implantation size (Table 1).

3.1.2 Aqueous and alcohol extracts

Oral administration of MP-aq extract at 70 gm DWE/PD_{1-4} showed normal implantation sites. It was also noted that the number and size of the implantation sites was comparable to that of the control (Table 1).

All animals dosed daily from PD_1 to PD_4 with MP-EtOH extract (Hong Kong) in 28 gm DWE showed no implantation site, while a dose of 15.4 gm DWE was half effective. To determine on which day MP is most effective, oral doses of MP-EtOH extract (China) were given on various days before implantation. It is clear that MP is most effective when given during the peroid of PD_1 and PD_2. A lower dose at 5 gm DWE had no apparent anti-implantation activity. Table 2 showed that when 30 gm DWE of plant materials (effective on PD_{1-4}), equally distributed to 10 similar doses (3 gm DWE/day) administered during the

peroid of PD_1 to PD_{10} failed to suppress implantation. There was no significant difference in the mean number and size of the implantation sites.

3.1.3 Chloroform fraction

Further purification of MP-EtOH extract by partition between water and chloroform gave rise to 2 fractions. There was no anti-implantation activity in the aqueous fraction even at the highest dose level of 100 gm DWE. The anti-implantation activity was detected in the chloroform fraction. Oral administration of the chloroform extract at 20-33 gm DWE/PD_{1-4} rendered implantation impossible (Table 3).

3.1.4 Butanol fraction

Aqueous fraction obtained from partition between butanol and water of the MP-EtOH extract at a dose as high as 94.4 gm DWE was ineffective in suppressing implantation. In contrast, the butanol fraction showed a dose related anti-implantation activity (Table 4). Significantly smaller implantation sites (range from 0.34-0.37 cm) were evident in females treated with butanol extract at a dose level of 60 gm DWE.

3.1.5 Alcohol extract of the aerial parts

The total alcohol extract of leaves and twigs of MP was toxic to the animal at a dose of 40 gm DWE/4 days. At this toxic dose level there was no effect on

Table 1 Anti-implantation Effects of Vehicle, MP Aqueous Root Extract and Alcohol Extract of the Aerial Parts

Treatment Total Dose in gm DWE (Plant Source)[a]	Date of Drug Administration	No. Animal with no Implantation Site/ Animal Used	Implantation Sites at Autopsy[b] Mean No. ± S.E. (n)	Mean Size ± S.E. (n)[c]
Control	-----	0/8 (0%)	11.40 ± 0.38 (8)	0.46 ± 0.01 (91)
Vehicle[d]	PD_{1-4}	0/4 (0%)	12.50 ± 0.50 (4)	0.44 ± 0.01 (50)
Aqueous root extract 70.0 gm (C)	PD_{1-10}	0/10 (0%)	13.60 ± 0.60 (10)	0.46 ± 0.01 (136)
MP-Aerial (HK)				
30.0 gm	PD_{1-4}	0/3 (0%)	9.70 ± 1.50 (3)	0.42 ± 0.02 (29)
41.6 gm	PD_{1-4}	0/8 (0%)[e]	12.30 ± 0.00 (8)	0.46 ± 0.01 (98)

a Plant materials were obtained either locally (HK) or from China (C)

b Autopsy was carried out on PD_8

c Length of the long axis of implantation sites in cm

d Received 1 ml of vehicle daily (ig, Ethanol/Tween 80/Water : 2/1/7)

e One animal died on PD_2, two died on PD_3

Table 2 Anti-implantation Effects of MP Alcohol Extract

Treatment Total Dose in gm DWE (Plant Source)[a]	Date of Drug Administration	No. Animal With no Implantation Site/ Animal Used	Implantation Sites at Autopsy	
			Mean No. ± S.E. (n)	Mean Size ± S.E. (n)
Control	-----	0/8 (0%)	11.40 ± 0.38 (8)	0.46 ± 0.01 (91)
15.4 gm (HK)	PD_{1-4}	2/4 (50%)	3.00 ± 0.00 (2)*	0.32 ± 0.03 (6)*
28.0 gm (HK)	PD_{1-4}	8/8 (100%)	0*	0*
76.0 gm (CM)	PD_{1-4}	3/3 (100%)	0*	0*
20.0 gm (C)	PD_{1-4}	4/4 (100%)	0*	0*
5.0 gm (C)	PD_{1-2}	0/4 (0%)	12.00 ± 1.08 (4)	0.44 ± 0.03 (48)
10.0 gm (C)	PD_{1-2}	0/5 (0%)	5.20 ± 0.73 (5)*	0.24 ± 0.01 (26)*
15.0 gm (C)	PD_{1-2}	5/5 (100%)	0*	0*
20.0 gm (C)	PD_{1-2}	6/6 (100%)	0*	0*
20.0 gm (C)	PD_{1-10}	0/8 (0%)	9.00 ± 1.51 (8)	0.42 ± 0.03 (72)
30.0 gm (C)	PD_{3-4}	0/4 (0%)	11.10 ± 1.50 (4)	0.38 ± 0.03 (44)*

a Plant materials were obtained locally (HK), from Chiangmai (CM) or from China (C)

* $P < 0.01$ by Student "t" test

Table 3 Anti-implantation Effects of MP Chloroform Extract

Treatment [b] Total Dose in gm DWE (Plant Source)	No. animal with no Implantation Site/ Animal used (%)	Implantation Sites at Autopsy Mean No. ± S.E. (n)	Mean Size ± S.E. (n)
Control	0/8 (0%)	11.4 ± 0.38 (8)	0.46 ± 0.01 (91)
Chloroform Fraction[a]			
33.2 gm (HK)	4/4 (100%)	0*	0*
70.8 gm (HK)	4/4 (100%)	0*	0*
20.0 gm (C)	5/5 (100%)	0*	0*
40.0 gm (C)	7/7 (100%)	0*	0*
Aqueous Fraction[a]			
60.0 gm (C)	0/2 (0%)	12.50 ± 2.50 (2)	0.43 ± 0.03 (25)
100.0 gm (C)	0/2 (0%)	11.00 ± 0.00 (2)	0.32 ± 0.02 (22)*

a Alcohol Extract was partitioned with chloroform and distilled water to form the chloroform and aqueous fractions

Plant materials were obtained locally

b The animals were dosed on PD_{1-4}

* $p < 0.01$, student "t" test

Table 4 Anti-implantation Effects of MP Butanol Fraction[a]

Treatment[c] Total Dose in gm DWE	No. Animal with No Implantation Site/ Animal Used (%)	Implantation Sites at Autopsy	
		Mean No. ± S.E. (n)	Mean Size ± S.E. (n)
Control	0/8 (0%)	11.40 ± 0.38 (8)	0.46 ± 0.01 (91)
Butanol Fraction[b]			
30.6 gm	0/3 (0%)	9.70 ± 2.30 (3)	0.37 ± 0.02 (29)
50.4 gm	3/5 (60%)	4.50 ± 1.50 (2)*[d]	0.34 ± 0.02 (9)*
60.0 gm	4/4 (100%)	0*	0*
Aqueous Fraction[b]			
48.0 gm	0/5 (0%)	11.20 ± 0.70 (5)	0.45 ± 0.03 (56)
94.4 gm	0/4 (0%)	10.00 ± 1.10 (4)	0.42 ± 0.13 (40)

a Plant Materials were obtained locally

b Alcohol Extract was partitioned with butanol and distilled water to form the butanol and aqueous fractions

c Animals were dosed on PD_{1-4}

* $P < 0.01$ by Student "t" test

Table 5 Confirmation of The Anti-implantation Effects of MP Alcohol Extract[a]

Treatment[b] Total Dose in gm DWE	No. Animal with No Implantation Site/ Animal Used (%)	Implantation Sites at Autopsy Mean No. ± S.E. (n)	Mean Size ± S.E. (n)
Vehicle	0/11 (0%)	13.40 ± 0.46 (11)	0.46 ± 0.01 (147)
MP-EtOH			
32 gm	13/13 (100%)	0*	0*
36 gm to 52 gm	6/6 (100%)	0*	0*

a The work was performed in the Worcester Foundation for Experiment Biology. Charles River CD rat was used. Plant extract was obtained from Hong Kong and plant materials were obtained locally.

b Animal was dosed on PD_{1-4}

* $P < 0.01$ by Student "t" test

implantation (Table 1).

Work performed in Worcester Foundation for Experimental Biology (Table 5) confirmed that administration of 32 to 52 gm DWE of MP-EtOH was effective in preventing implantation.

3.2 Effects of MP on delayed implantation

Animals were treated on PD_1 and PD_2 with a total dosage of 10 gm DWE; this dosage could reduce the size of implantation sites observed on day 8 of pregnancy ($p<0.01$), but allowed pregnancy to occur (Table 6). The first signs of implantation were delayed for some 24 hrs. In control animals the blue reaction become visible in some rats by about 1200 hrs on PD_5 and was positive in all the animals studied by 1500 hrs. In treated animals, however, the blue reaction did not become visible in some rats until midmorning on PD_6. By 1400 hrs, there was a positive blue reaction in all the rats autopsied at this time.

3.3 Uterotrophic effect of MP

The uterotrophic effect of MP-EtOH extract on spayed rats is shown in Table 7. When animals were treated daily (for 3 days) with 1 gm DWE of MP-EtOH extract there was a significant increase in uterine weight. Subcutaneous injection of 2.5 μg estradiol-17β from day 1 to 3 also stimulated the uterotrophic response. A significant difference in uterine weights was observed between the estradiol-17β

Table 6 Effects of MP Alcohol Extract (MP-EtOH) on Delayed Implantation

Treatment[a]	Date of Autopsy[b] (time)	No. of Rats Showing Positive Blue Reaction/No. of rats Used
Control	PD_5 (0600-0800)	0/2
	PD_5 (0800-1000)	0/2
	PD_5 (1200-1400)	2/2
	PD_5 (1500-1700)	2/2
	PD_5 (1800-1900)	5/5
Test	PD_5 (2200-2400)	0/2
	PD_6 (0700-0900)	0/2
	PD_6 (0900-1000)	1/2
	PD_6 (1000-1200)	1/4
	PD_6 (1400-1600)	2/2

a Control group and test group received 1 ml of vehicle or 5 gm DWE of MP-EtOH respectively, (PD_{1-2}, ig)

b Animal was autopsied 10 to 15 minutes after the injection (iv) of 0.2 ml 0.3% Pontamine Sky Blue in saline

Table 7 The Uterotrophic Effect of MP Alcohol Extract (MP-EtOH) and Estradiol-17β on Castrated Rat

Treatment[a] Dose/Day	Uterine Weight (gm) $\bar{X} \pm$ S.E. (n)
Vehicle[b] 0.1 ml	45.38 ± 0.69 (5)
Estradiol-17β 2.5 μg	58.58 ± 1.33 (5)*
MP-EtOH 1 gm DWE	77.04 ± 2.04 (5)*

a The animal was dosed once daily from day 1 to day 3, autopsy was carried out 24 hr after the last dosage

b Vehicle contains Ethanol/Tween 80/Water : 2/1/7

* $P < 0.01$

and vehicle treated animals with a P value less than 0.01.

3.4 Effects of MP on Stimulating Vaginal Cornification

Spayed animals showed a prolonged leukocyte vaginal smear during the experimental peroid. In contrast, an estrus smear is typical on the third to fourth day of MP treatment. Smear patterns with dense cornifed epithelial cells were found in virtually all of the rats treated daily with 2 to 5 gm DWE of MP-EtOH extract.

3.5 Effects of Estradiol-17β and MP on the levels of Estrogen Receptor and Uterine Growth

3.5.1 Nuclear Receptor

No significant difference was observed in the levels of nuclear receptor site between all controls and estradiol-17β treated animals in all treatment groups (24 hr, 1 injection; 48 hr, 1 injection and 48 hr, 2 injections). On the other hand, the pattern of nuclear receptor level of both MP and MP+E_2 groups were similar, administration of MP or MP+E_2 resulted in a moderate increase of nuclear receptor sites 24 hrs after, followed by a slight depletion in receptor level as determined at 48 hrs. In 48 hr, 2 injections group, there was a 4 fold increase in the amount of nuclear binding sites in rats treated with MP and MP+E_2, as compared to the controls (Fig.2). However, no additive effect was observed in animals treated with MP+E_2.

3.5.2 Cytoplasmic receptor

Following a single treatment of MP, E_2, or

Fig.2 The effect of E_2, MP and MP+E_2 on nuclear estrogen receptor content of the uterus. Immature rats were dosed with A: saline □, B: 2.5 µg E_2 ⁙, C: 2 gm DWE MP ▤, D: MP+E_2 (E_2, µg and MP, 2 gm DWE) ▤. Rats were either killed 24 and 48 hr after treatment or were reinjected at 24 hr and killed at 48 hr. The quantity of cytoplasmic estrogen receptor was determined by the ^3H exchange assay. (Specific activity of ^3H estradiol was 101.7 Ci/mmole; counting efficiency was 33%). The vertical bars represent the mean ± S.E. of 3 determinations, with 3 rats per determination. * $p < 0.05$; ** $p < 0.01$.

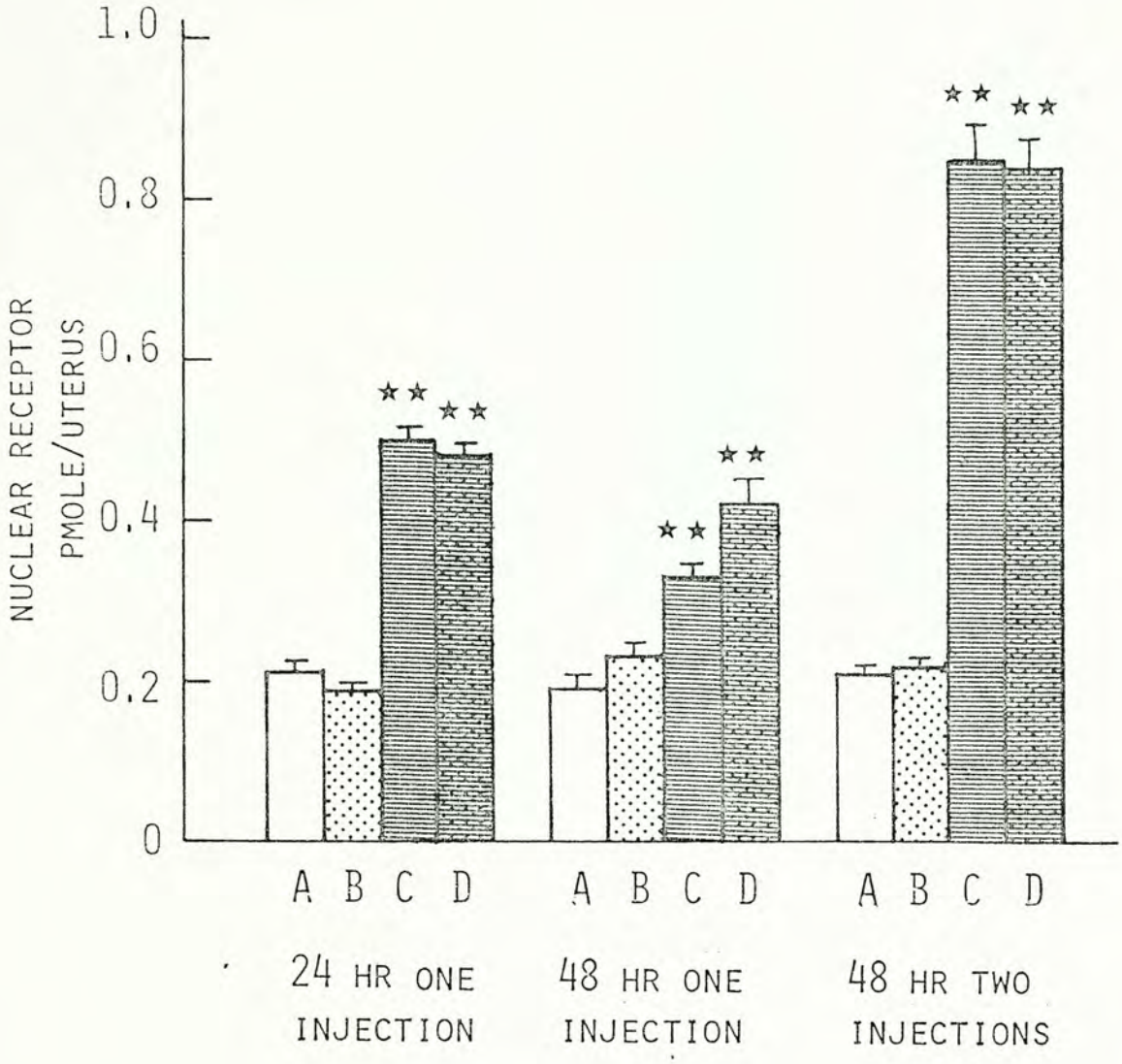
NUCLEAR RECEPTOR
PMOLE/UTERUS
1.0
0.8
0.6
0.4
0.2
0
A B C D
A B C D
A B C D
24 HR ONE INJECTION
48 HR ONE INJECTION
48 HR TWO INJECTIONS

FIG. 2

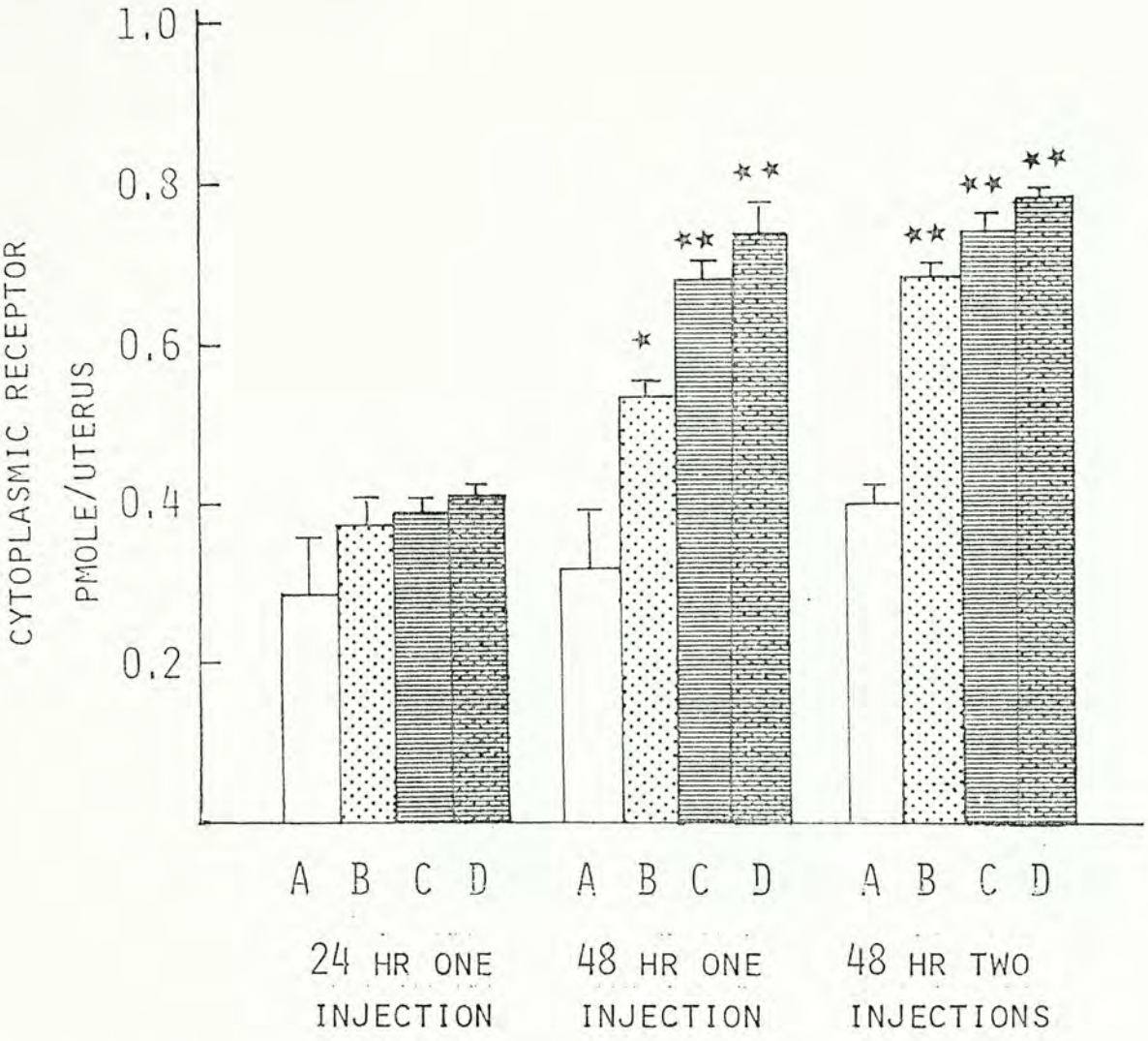

FIG.3 The effects of E_2, MP and MP+E_2 on cytoplasmic estrogen receptor content of the uterus. Immature rats were dosed with A: saline; B: E_2; C: MP; D: MP+E_2. The quantity of cytoplasmic estrogen receptor was determined by the ^3H estradiol exchange assay. The vertical bars represent the mean $\pm$ S.E. of 3 determinations, with 3 rats per determination. ★ $p < 0.05$; ★★ $p < 0.01$.

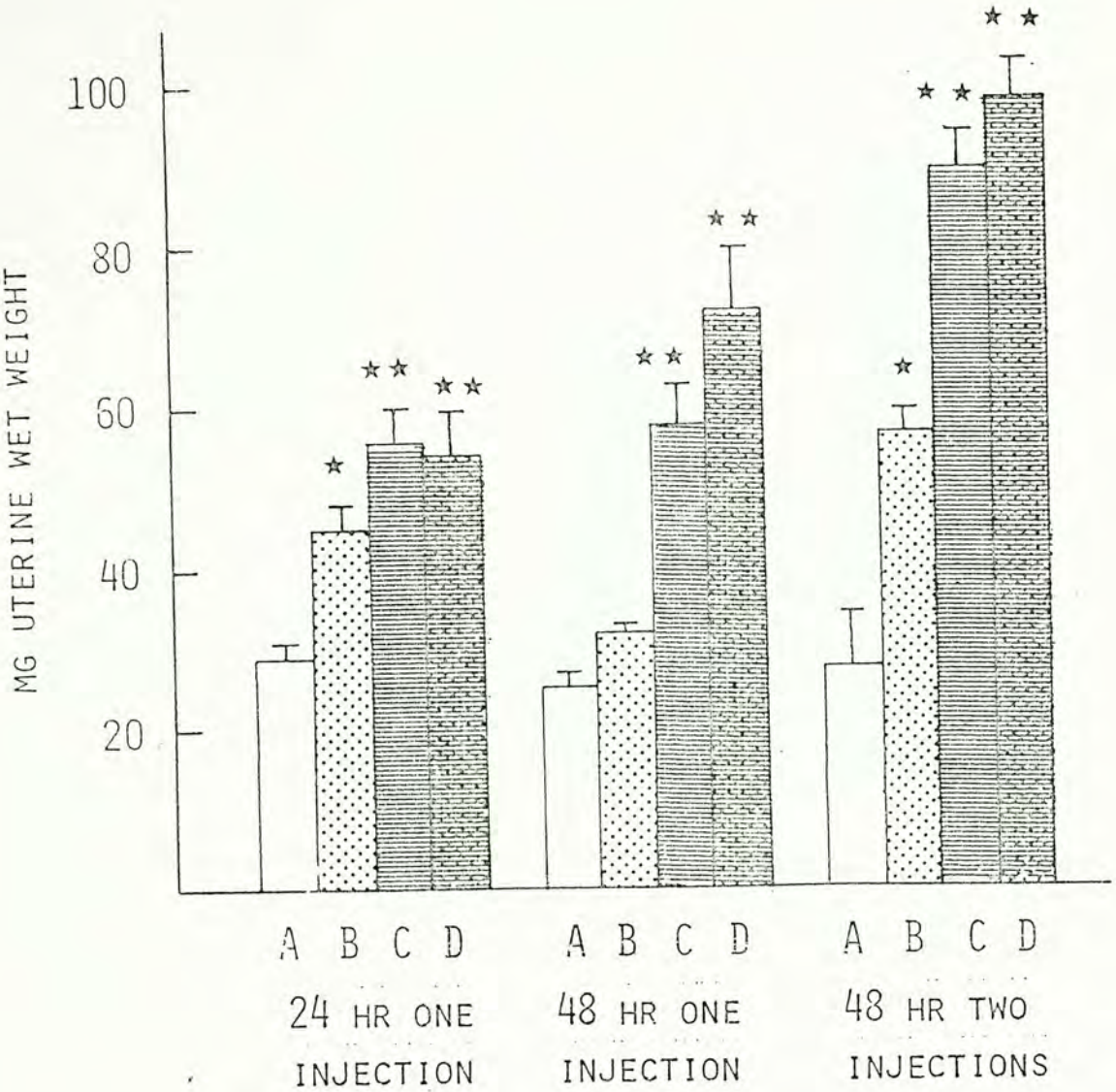

FIG.4 The effects of E_2, MP and MP+E_2 on uterine wet weight. Immature rats were dosed with A: saline; B: E_2; C: MP; D: MP+E_2. Rats were either killed 24 and 48 hr after treatment or were reinjected at 24 hr and killed at 48 hr. The uteri were weighed. The vertical bars represent the mean $\pm$ S.E. of 9 animals. $\star\ p < 0.05$; $\star\star\ p < 0.01$.

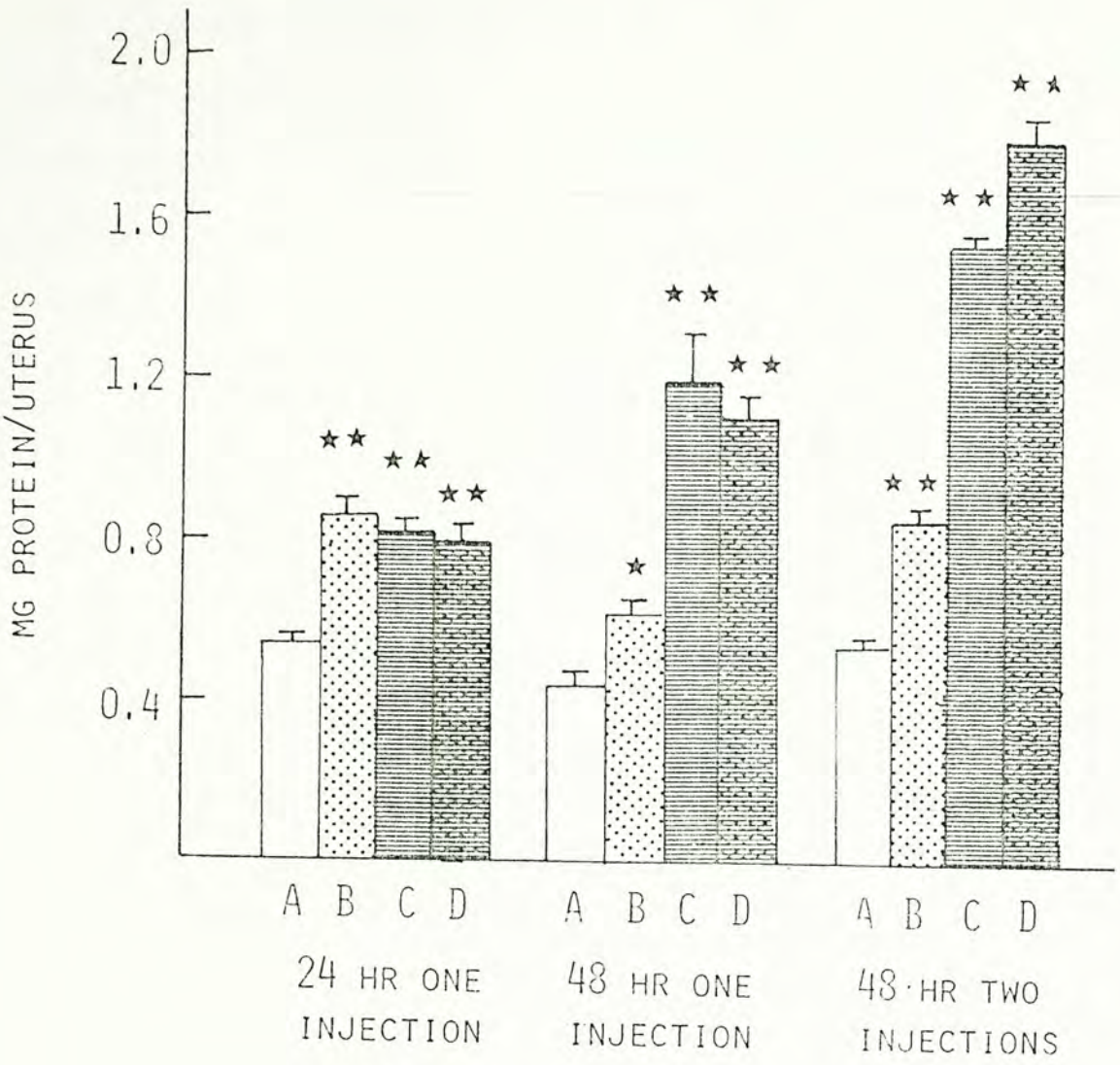

FIG.5 The effects of E_2, MP and MP+E_2 on cytosol protein content of the uterus.
Immature rats were dosed with A: saline, B: E_2, C: MP; D: MP+E_2. The cytosol protein content was determined by the Folin Ciocalteau test. The vertical bars represent the mean ± S.E. of 9 animals. ✱ $p < 0.05$; ✱✱ $p < 0.01$.

MP+E_2, the amount of cytoplasmic estrogen receptor was slightly increase by 24 hr. A further enhancement was observed at 48 hr. MP and MP+E_2 showed equal potency when given once or twice within 48 hrs. In the estradiol treated group, there was a 28% increase by the two dosing schedule (Fig.3).

3.5.3 Uterine weight and cytosol protein content

Animals dosed with MP, E_2, or MP+E_2, showed a uterotrophic response (significant increase in both cytosol protein content and uterine weight) in all treatment group. Treatment with MP+E_2 is superior to that with E_2 or MP alone in the stimulation of uterine growth. Therefore, following a single or double treatment of MP+E_2 by 24 hrs, agonistic effect is observed (Fig.4 & 5).

3.6 Effects of MP on decidual formation

The effect of MP-EtOH on decidual formation is presented in Table 8.

After 10 to 40 gm DWE of MP-EtOH extract given on PSP_{0-3}, none of the fourteen hooded females had decidualized uteri following oil injection, as opposed to all 6 of the vehicle treated females. That MP had affected the normal growth of the pseudopregnant uterus was evidenced by the drastically reduced mean uterine weight difference between the two horns in the test group.

The same held true for the unilateral pregnant animals. It was obvious that in the control group significant weight increase due to deciduoma formation took

Table 8 The Effect of MP Alcohol Extract (MP-EtOH) on The Decidual Reaction in Pseudopregnant and Unilateral Pregnant Rats[a]

Status	Total Dose gm DWE	Date of Administration	% with Deciduoamta (No. of Animal Used)	Response (mg)[b] $\bar{X} \pm$ S.E.
Pseudo-pregnant	0 (control)	-----	100% (6)	1130.50 ± 239.00[+]
	10	PSP_{0-3}	0% (5)	5.01 ± 6.33[*]
	20	PSP_{0-3}	0% (5)	3.28 ± 8.20[*]
	40	PSP_{0-3}	0% (14)	2.28 ± 5.53[*]
	40	PSP_{4-8}	100% (3)	1429.00 ± 167.00[+]
Unilateral Pregnant	0	------	100% (3)	1386.40 ± 4.75[+]
	40	PD_{1-4}	0% (4)	4.25 ± 6.65[*]

a Psuedopregnancy was induced by mechanical stimulation of the cervix, while unilateral pregnant rat was prepared by ligating the left oviduct. The stimulus for deciduoma initiation was provided on PSP_4 by injection of 0.05 ml arachis oil into the uterine lumen.

b Response of the psuedopregnant group was represented by the weight difference between two uterine horns. In the unilateral pregnant group, weight difference between the left uterine horn of the unilateral pregnant rat and the mean weight of the right horn of the control pseudopregnant rats (5.22 ± 4.38 mg (6)) was measured as the response.

+ $P > 0.05$ by Analysis of Variance

* $P < 0.001$ by Student "t" test

place in the left horn whereas in the test group there was no significant difference in weight between the two horns. When the extract was given on PSP_{4-8} (i.e. after the oil injection), it was ineffective in inhibiting decidualization.

The weight of all oil injected horns which had responded formed a homogenous population as shown by an analysis of variance ($p>0.05$). This agreed with the findings of Finn & Emmens (1961).

The control animals showed a prolonged leukocyte vaginal smear during the experimental peroid. In contrast, a proestrus smear is typical on PSP_3 of the treated group. Within the next 24 hrs (i.e. PSP_4), a typical estrus pattern is usually revealed in the vaginal smear.

3.7 Effects of MP on the changes of serum steroid hormone concentration

Oral administration of MP-EtOH extract at a dose level of 36 to 52 gm DWE/PD_{1-4}, caused significant changes in the steroid concentrations (Table 9). This dosage had been previously tested to be effective in suppressing implantation. A significant drop in serum estradiol level was observed on PD_3 and PD_5 ($p<0.05$). On the other hand, serum progesterone level was drastically enhanced only on PD_5 ($p<0.01$), as compared to vehicle-treated controls on the corresponding days of pregnancy.

By contrast, in rats after treated with 32 gm DWE, a dose which proved to be effective in suppress-

Table 9 Temporal Changes of Serum steroid concentration in Rats treated with Vehicle or MP Alcohol Extract

Treatment[a]	Day of Pregnancy	Estradiol-17 (g/ml) $\bar{X} \pm$ S.E. (n)	Progesterone (g/ml) $\bar{X} \pm$ S.E. (n)
Vehicle	3	32.2 ± 4.7 (7)	58.2 ± 10.5 (7)
MP-EtOH 32 gm DWE 36 to 52 gm DWE	3	17.4 ± 2.1 (5)*	114.1 ± 27.8 (5)
Control	4	28.4 ± 4.2 (5)	90.5 ± 7.8 (5)
MP-EtOH 32 gm DWE 36 to 52 gm DWE	4	18.3 ± 3.5 (5)	103.4 ± 8.1 (5)
Control	5	38.3 ± 3.9 (7)	86.3 ± 11.7 (7)
32 gm DWE 36 to 52 gm DWE	5	24.6 ± 2.8 (4)*	256.0 ± 51.2 (4)**

a The animal was dosed on PD_{1-4}. Blood sample was collected during 0900 to 1000 hr on PD_{3-5}

* $P < 0.05$ by Student "t" test

** $P < 0.01$ by Student "t" test

ing implantation, serum estradiol level was not altered. However, changes in the progesterone level were observed. The hormone increased significantly on PD_5 ($p<0.05$, Table 9).

3.8 Effects of exogenous estradiol-17β on MP treated rats

Results indicate that a supplement of a small dose of estradiol (2.5 or 0.2 μg) to MP treated rats on PD_2 or PD_4, failed to abolish the delay of ovum implantation (Table 10).

3.9 Effects of MP on fetal sex ratio

Results on Table 11 showed that the plant extract had no adverse effect on either morphogenesis or fetal sex ratio. All pups were found to be in normal shape and good health.

3.10 Effects of MP on subsequent pregnancy

In the rats tested for subsequent pregnancy, the number of implantation sites ranged from 10 to 15, with a mean of 13.5 to 12.2. The size of the implantation sites ranged from 0.42 to 0.46 cm, with a mean length of 0.44 to 0.46 cm. Both the number and size of the implantation sites are comparable to that of the controls ($p>0.1$, Table 12).

Table 10 Effects of Exogenous Estradiol-17β on MP Treated Rat

Treatment	Date of Estradiol-17β Injection (hr)	No. of Rats with Implantation Site/No. of rat Treated
2.5 μg Estradiol	PD_4 (1100)	4/4
MP[a]	--------	0/4
MP + 2.5 μg Estradiol	PD_4 (1100)	0/5
MP + 2.5 μg Estradiol	PD_2 (1100)	0/2
MP + 0.2 μg Estradiol	PD_2 (1100)	0/2
MP + 2.5 μg Estradiol	PD_2 (1600)	0/2
MP + 0.2 μg Estradiol	PD_2 (1600)	0/2

a Total dosage of 32 gm DWE MP-EtOH was administered intragastrically on PD_{1-4}

Table 11 Effects of MP Alcohol Extract on Fetal Abnormality and Sex Ratio of The Offspring

Treatment[a]	Litter Size $\bar{X} \pm$ S.E. (n)	Sex of Fetuses		Remarks
		No. Male $\bar{X} \pm$ S.E. (n)	No. Female $\bar{X} \pm$ S.E. (n)	
Control	10.6 ± 0.50 (10)	4.8 ± 0.39 (10)	5.8 ± 0.53 (10)	All pups were found to be in normal shape and good health
Test	9.8 ± 0.54 (4)	4.75 ± 0.70 (4)[b]	5.25 ± 0.30 (4)[b]	All pups were found to be in normal shape and good health

a Test group received 5.0 gm MP alcohol extract on PD_{1-2}

b No significance difference was observed between the control and test group, $P > 0.1$ by Student "t" test

Table 12 Effects of MP Alcohol Extract (MP-EtOH) on Subsequent Pregnancy

Treatment[a]	Duration of Sterile Peroid (Day)[b] $\bar{X} \pm$ S.E. (n)	No. of Implantation Site $\bar{X} \pm$ S.E. (n)	Size of Implantation Site $\bar{X} \pm$ S.E. (n)
Vehicle	0 (14)	12.93 ± 0.51 (14)	0.52 ± 0.02 (182)
Test	15.43 ± 0.30 (7)	12.86 ± 0.63 (7)[c]	0.45 ± 0.01 (91)[c]

a Vehicle group received 1 ml vehicle (Ethanol/Tween 80/Water : 2/1/7)
Test group received a total dosage of 20 gm DWE MP alcohol extract
Autopsy was carried out on PD_8

b The duration of PSP was determined by examining the vaginal smear everyday morning and the day of appearance of proestrus smear was designated as the last day of PSP

c No significant difference between the vehicle and test group, $P > 0.1$ by Student "t" test

4 DISCUSSIONS

4.1 Suitability of the animal model

4.1.1 Animal type

There are evidences that great differences exist in the response and sensitivity to the same drug when tested in different species of animals (Harper, 1972). Therefore, a plant extract should be tested on at least two animal models for its anti-implantation activity. Much of the investigations on contraceptive screening have been done on rodents. Mouse, however, is not particularly suitable for this purpose because it is peculiarly sensitive to the effects of toxic substances. As little as 18 hrs of starvation during early pregnancy can prevent implantation in almost all mice (McLure, 1966). Thus, using mice for such work can lead to many false positive results. Since the albino rat is relatively economical to raise and its reproductive physiology the best understood among many laboratory animal, this species offers the first choice for primary screening of potential contraceptive agents.

Nearly all contraceptive compounds had first been tested in the rat or mouse, which provided sufficient interest to initiate studies in nonhuman primates (Kamboj *et al.*, 1970). However, the endocrinology of pregnancy of rodents and primates are quite different. For

example, plasma estrogen levels are low for a day or two after ovulation in both species, but with the exception of a brief estrogen surge on day 4 of pregnancy, the levels of estrogens remain relatively low and stable in the rat (Yoshinaga *et al.*, 1969). In contrast, estrogens increase during the luteal phase of the primate cycle and continue to rise as placental production of this steroid increases. Thus while estrogen excess is a nonphysiological situation in rat pregnancy, it is a normal endocrine function of human and nonhuman primate pregnancy (Hodgen *et al.*, 1972; Vande Wiele *et al.*, 1970). The comparative endocrinology of pregnancy in primates and rodents always warrants discrete interpretation and extrapolation of results from rats in contraceptive screening to human application.

4.1.2 Route of administration

Oral dosing is applied in the present experiment since it is a convenient and safe route that is widely used. Because administration of most drugs to humans is most easily achieved by the oral route, the majority of chemical agents tested for contraceptive activity have also been administered orally. However, even the drug can be absorbed through the digestive tract, it may cause irritation to the gastrointestinal tract or being inactivated by the digestive enzymes. Under these circumstances, other routes of administration, e.g. subcutaneous,

intravenous or intramuscular, are more preferable. In any case, at least 2 routes (one of which should be oral route of administration) should be tried.

4.1.3 Dose schedule

Since a drug can act on different stages of gestation, the first step to screen the anti-implantation property of a plant extract shall cover the entire pre-implantation stages. These include a time lapse between fertilization and implantation, i.e. between day 1 and 4 of pregnancy in the albino rat (Lau *et al.*, 1973).

4.1.4 Preparation of dosing extract

Hydrophobic extracts, such as those prepared with low polarity solvents (ethanol, butanol and chloroform), are almost insoluble in distilled water. For these types of extracts, alcohol and tween 80 serve as a good solubilizer and a suspending agent respectively, but toxicity problems may involve. It had been concluded that no more than 1/4 of the LD_{50} of a vehicle should be used in pharmacological and/or toxicological experiments (Bartsch *et al.*, 1976). The LD_{50} of ethanol and tween 80 for albino rat are 13.0 and 30.0 ml/kg body weight, respectively (Berezovskaya & Rudzit, 1976). The amount applied in preparation of the dosing extract of MP were less than one tenth of their LD_{50} (0.8 ml/kg body weight for ethanol and 0.4 ml/kg for tween 80).

However, it is well known that many of the common vehicles can either enhance or diminish the biological activity of compounds being evaluated (Bartsch *et al.*, 1976). In order to eliminate the effect of vehicle on the anti-implantation potency of the dosing extracts, another type of vehicle, tragacanth powder (tragacanth + starch + acacia), 10% (w/v), was used as suspending agent. As the dosing extract prepared by tragacanth powder was equally effective in suppressing pregnancy, it is likely that the ani-implantation effect of the dosing extracts were associated with the inherent properties of the plant materials.

4.1.5 End point detection

Implantation swellings appear along the uterine horns by day 6 and prominent implantation sites can be distinguished easily when autopsied on day 7. The size of the implantation sites is important in determining the time of implantation especially when the measurement is taken 2-3 days after implantation of the blastocysts. If the size of implantation site is smaller than normal, as in the case of MP treated rats, the drug might have caused a delayed implantation (Yoshinaga *et al.*, 1979).

A very early uterine endometrial change at the site of ovum implantation is the increase in the permeability of the local capillaries. The change

can be easily visualized by the accummulation of intravenously injected macromolecular dyes, such as pontamine sky blue. This dye will bind to serum albumin and escape only through the capillaries with an increased permeability. The implantaton sites are recognized as blue spots in the uterus (Psychoyos, 1971). Therefore, this method serves as a useful technique for determining the timing of ovum implantation.

4.2 Anti-implantation effect of MP extracts

The aqueous extract, when given orally at a dose level of 70 gm DWE, failed to prevent implantation in all treated rats (n=10). The effect of the alcohol extract, as reflected by the decrease of the number and size of the implantation sites represents a dose dependent response, indicating that the effect of the extract on implantation in the animal is not "all or none" in nature. Oral administration of the alcohol extract in a minimum of 15 gm DWE was effective in preventing implantation. Purification by partition between chloroform and water revealed that the active principle is readily soluble in chloroform. Treatment with aqueous fraction at a total dose of as high as 100 gm DWE did not affect implantation (Table 3). The minimum effective dosage of the chloroform fraction is 20 gm DWE, which is comparable to that of the ethanol extract. Butanol fraction obtained from partition between butanol and

water is also effective, but the minimal effective dosage of the butanol fraction is 60 gm DWE. The decrease in potency may be due to the loss of active ingredient(s) or insufficient extraction into the butanol phase during the butanol/water partition step.

In summary, the active principle is probably nonpolar in nature, and can be completely extracted by chloroform.

4.3 Estrogenic activity

4.3.1 Uterotrophic response

Like the effect of estrogen, a sustained increase (48 hrs) in uterine weight was observed in intact immature rats orally dosed with MP(Fig5). With the MP extract, an antifertility effect is produced at a dose level (15 gm DWE) which is greater than that in stimulating uterine growth (2 gm DWE). The same property is observed in many of the conventional estrogens (Pincus *et al.*, 1964).

4.3.2 Vaginal cornification

MP acts as a potent estrogen and is evidenced by the stimulation of vaginal cornification in pseudopregnant rats. It is also effective in producing vaginal cornification in ovariectomized rats at a dose level of 2 gm DWE only.

4.3.3 Estrogen or antiestrogen ?

The effects of exogenous estrogen on fertility has been extensively studied (Parkes & Bellerby,

1926; Dreisbach, 1959; Greenwald, 1961) and it was stated that any compound with estrogenic activity may have antifertility effect if taken at a particular time soon after mating (Chang & Yanagimachi, 1965).

However, there seems to be a high correlation between estrogenic and antiestrogenic activity (Emmens *et al.*, 1964). It is recognized that under certain circumstances, antiestrogens also exert an estrogenic effect on uterus, for example, nofoxidine, can increase uterine weight when injected into immature rat, but when it is used simultaneously with typical estrogen agonist, it display an antagonistic effect Katzenellenbogen & Ferguson, 1975). This correlation has made it difficult to decide whether the antifertility activity shown by many of these antiestrogens is due to their estrogenic or antiestrogenic action (Martin *et al.*, 1963; Emmens *et al.*, 1964). It was suggested that antiestrogens antagonize estrogen-induced uterine growth as á result of their failure to stimulate the replenishment of the cytoplasmic estrogen receptor (Clark *et al.*, 1974). Nafoxidine causes accummulation and long term retention of the nuclear receptor complex. However, this retention is not accompanied by the usual replenishment of cytoplasmic receptor, as in the case after estradiol treatment. This failure to stimulate the replenishment of cytoplasmic receptor may render the uterus insensitive to subsequent injection of estrogen, and therefore, antagonism can be observed.

In order to evaluate the agonist/ antagonist properties of MP, the effects of MP and MP+E_2 on nuclear and cytoplasmic receptor content and on uterine growth are studied. In the present experiment, the animal received serial doses of MP at 24 hr intervals. This method has been used routinely by many investigators, and that estrogen antagonism can be observed under these conditions (Callantine *et al.*, 1966; Clark *et al.*,1977). As the receptor antagonist complex fails to cause the replenishment of cytoplasmic receptor, when the animal receives a second dose of estrogen of this time, the uterus is unresponsive in the animals that received the antagonist but is highly responsive in the estradiol treated animals. Therefore, the uterus continues to grow in the estrogen treated rat and remains unstimulated in the antagonist treated animal.

It was demonstrated by Katzenellenbogen (1975) that following a single injection of E_2 (5 μg), the level of nuclear estrogen receptor was maximal by 1 hr, declined rapidly to levels only slightly above control by 4 hrs and remained at this level until as long as measured thereafter (42 hrs). The present data confirms the observations of Katzenellenbogen; there was no significant difference in the level of nuclear receptor sites between the controls and E_2 treated animals, whereas a prominant increase in cytoplasmic receptor was observed (Fig 2 & 3).

In contrast to the short nuclear residency of the estradiol-receptor complex, MP causes nuclear retention of the receptor for long periods of time (48 hrs, fig 2). This stimulation in uterine growth is superior to that of estradiol in magnitude and the length of time of growth stimualtion (Fig 4 & 5). Therefore, MP is similar to nafoxidine, a long acting estrogen (Clark *et al.*, 1972; 1973), causing growth stimulation and nuclear retention of the receptor which exceed that induced by estradiol (Clark *et al.*, 1973; 1974; Rochefort *et al.*, 1972). It is likely that the active principle(s) of MP are cleared slowly from the body, and cause nuclear retention of the receptor for much longer periods than estradiol.

However, unlike nafoxidine, MP nuclear receptor complex stimulates full replenishment of cytoplasmic receptor, whereas the nafoxidine receptor complex fails to cause the replenishment by 24 hrs (Clark *et al.*, 1972; 1973). When the animal receives a second dosage of MP after 24 hrs, the uterus continues to grow and no estrogen antagonistic effect was observed in the MP treated animals, after a single or a double treatment. The quantity of cytoplasmic receptor in MP or MP+E_2 treated rats were elevated by 48 hrs (1 or 2 injections). This replenished cytoplasmic receptor is then free to interact with estrogen to form receptor hormone complexes which bind to nuclear sites and cause a second cycle of uterine growth stimulation.

4.4 Action mechanisms

Laboratory evidence reveals that MP, being a potent estrogen, is an effective interceptive agent if given during the immediate post-ovulatory period. It is likely that MP may act through the following routes: acceleration in tubal transport of ova, a direct blastotoxic effect, suppression of luteal function, or an imbalance in the hormonally directed progression of endometrial changes necessary for implantation.

4.4.1 Effects of MP on oviductal transport

Plant extracts that are effective on PD_{3-4} may have some effects on the uterine environment which becomes hostile to the developing embryos, and those only effective on PD_{4-5} will cause hormonal disturbance, resulting in a failure of implantation process, since MP is effective only on PD_{1-2}, it would appear that egg transport is being affected. It was reported that estrogen given during the peroid of tubal transport of eggs can be extremely effective as postcoital antifertility agents. Once the egg has entered the uterus, more estrogen is required to prevent pregnancy, and after implantation, at least in primates, effectiveness is completely lost (Morris *et al.*, 1971; 1973). It may be that the hormone exerts its maximum effect on oviduct motility and egg transport. In all species so far examined, estrogen has been shown to disturb egg

transport (Greenwald, 1967; Humphrey, 1968; Harper, 1967; Banik & Pincus, 1964).

In order to distinguish whether the drug acts directly on the embryo or the anti-implantation effect is mediated through the adverse effect of the drug on the uterus, the ovum transfer technique may be applied (Smith, 1968; Wu & Meyer, 1970). The main disadvantage of this technique is the technical skill required and the time comsumed to perform it, which makes large scale experiments very time consuming and difficult to control. The blastocyst is a very sensitive living organism and even under ideal circumstances, one rarely gets all the transplanted ova implanted. Owing to the technical difficulty of obtaining satisfactory percentage of ovum recovery in oviduct flushing, the possibility of MP acting on oviductal transport cannot be further confirmed.

4.4.2 Effects of MP on decidual formation

Besides the ovum transfer technique, however, a much simpler method, i.e. the use of an artificial stimulus to induce decidualization in the uterus, can be applied. By using this as a model, it is able to rule out any effect on the eggs, and also to get more accurate information on the time relationships of the sensitivity of the uterus to these substances. It has been shown in rats

that estrogen can have a direct inhibitory effect on implantation after the period of tubal transport, by preventing or delaying the increase capillary permeability of the uterus or by interfering with the ability of the stromal cells of the endometrium to undergo decidualization (Harper, 1968a).

Result shows that MP, in a dosage of 10 gm DWE, is effective in inhibiting decidual formation. Yet, it cannot be determined whether this effect on the uterus is responsible for the antifertility effect of MP because the effective dosage for inhibiting deciduoma formation (10 gm DWE) is considerably less than the dosage required to prevent implantation (15 gm DWE, compare table 2 & 8). This property is also observed in other estrogenic compounds, such as dienestrol (Harper, 1968a, diethylstilbestrol (Finn & Emmens, 1961), androstenedione (Harper, 1967) and clomiphene (Stapes, 1966). It is probable that the hormone conditions of pseudopregnancy and decidual cell production which is induced by cervical stimulation and oil injection of the uterus are not quantitatively the same as those of true pregnancy. Possibly the developing blastocyst supplies some stimulus to decidual cell production which is more resistant than the stimulus of oil injection to interference by hormones or similar substances (Finn, 1966). Therefore, a higher dose is required to inhibit

pregnancy than decidualization.

The weight of all oil injected horns which had responded formed a homogenous population as shown by analysis of variance ($p>0.1$). From this, it can be seen that the inhibition is all or nothing in nature and the compounds are inhibiting some mechanism which triggers the decidual response, rather than inhibiting the actual development of the decidual cells. Work by Finn (1966) showed two ways in which relatively small amounts of estradiol at certain stages can upset the sensitization of the uterus for implantation. This may account for the all or nothing nature of the inhibition. Suppression happens when either the level of estrogen at the time of the nidatory estrogen surge rises above the concentrations at which an inhibitory effect occurs, or if given before this time, premature sensitization is induced followed by a refractory state so that the natural surge is ineffective. In normal circumstances the small surge of estrogen just prior to implantation causes some change in the uterus necessary for the decidual response. This change is histamine release from the mast cells (Finn & Emmens, 1961). Administration of estrogen before this natural surge could initiate such a change prematurely, and ineffectively, because the egg would not have developed sufficiently to implant. In doing so it could deplete the uterus of the substance concerned,

perhaps histamine, thus rendering it unable to respond to the later endogenous surge of estrogen. Estrogens have been shown to cause release of histamine from the uterus and depletion of mast cells (Ferrando & Nalbandov, 1968).

4.4.3 Effects of MP on the changes in serum hormone levels

Results indicate that MP is effective in delaying implantation of the ovum. If these disturbance of early pregnancy are caused by or related to a hormonal imbalance, the effect of MP on pregnancy may be, at least, partly attributed to an indirect effect through the endocrine system. In order to investigate this possibility, serum concentrations of Δ^4P and E_2 were evaluated during the period of PD_{3-5}.

As shown in Table 9, serum levels of the two steroids, Δ^4P and E_2 in control rats are comparable to those reported by Yoshinaga (1979) on the corresponding days of pregnancy.

As the onset of this study, we assumed that delayed implantation in MP-treated rats might be due to a low E_2 level. This assumption was based on the well established endocrine background of delayed implantation in lactating pregnant rats; the delay is caused by low levels of E_2 (Krehbiel, 1941; Weichert, 1941; Yoshinaga, 1961). This was further confirmed by the results obtained from radioimmunoassay, that estradiol levels were lower

(significant on PD_3 and PD_5) in MP treated rats (in a dosage of 36 to 52 gm DWE) than in controls. On the contrary, for rats treated with 32 gm DWE of MP, a dose which was proved to be 100% effective in terminating pregnancy, however, no difference in serum E_2 levels was observed. This suggests that insufficient secretion of E_2 during early pregnancy may not be the cause of delayed implantation in MP treated rats.

In Table 10, results show that the anti-implantation action of MP cannot be inhibited to any extent by the administration of exogenous E_2. The doses of E_2 used (2.5 or 0.2 µg) are quite considerable and in rats treated with 2.5 µg E_2, implantation sites are found in 100% (4/4) of rats. The failure of E_2 administered on PD_2 or PD_4 to restore normal implantation, supports the view that the effect of MP at this time is not due to its E_2 depleting action.

There was an increase in serum Δ^4P levels on PD_3 and PD_5. However, the enhancement are again associated with the high dose treated group and a significant difference is only observed on PD_5. Therefore, it cannot be determined whether an increase of Δ^4P secretion is responsible for the delay in ovum implantation in MP treated animals.

4.5 Teratogenic effects of MP

4.5.1 Sex ratio

In addition to requirements of effectiveness, interception involves the risk that if taken in marginal doses or at marginal times, the compound may prove teratogenic. Teratogenesis has been noted with a variety of interceptive agents, such as U11100A, U11555A and parahydroxypropionphenone (Morris, 1970). However, no teratogenicity (other than virilization with androgens) has been observed with any steroid, including estrogen. MP, being a potent estrogen, also exhibits no adverse effect on the development of the fetuses.

Since gonadal abnormalities were observed in rabbit fetuses after *in vitro* treatments of fertilized eggs with E_2 and stilbestrol, it is of interest to study the effects of MP on abnormality of rat fetuses, and results indicate that no virilization effect is observed.

4.5.2 Subsequent pregnancy

Apparently normal mating and pregnancy occurred after the pseudopregnancy consequent of MP administration. That the sterile period induced a pseudopregnancy is confirmed by persistent leukocytic smear pattern. There thus appears to have no carryover of the sterilizing action of MP. This effect is similar to many of the estrogen analogues, as reported by G. Pincus (1964).

4.6 General discussions

In the present study, the anti-implantation activity of MP is evaluated. It is most effective when given on PD_{1-2} and is not active on PD_3 or thereafter. Half of the minimal effective dose required to suppress implantation is strong enough to stimulate vaginal cornification. Estradiol and estrone possess similar characteristics with MP: when given on PD_{1-2}, the two hormones cause retention of eggs in the oviduct and interfere with the uterine reaction (Edgren & Shipley, 1961). Hence, it is likely that the effect of MP on early pregnancy in rat is due to its inherent estrogenicity.

It is shown that MP not only influenced the endocrine system, but also inhibit the decidual formation and most likely interfere with oviductal transport. Therefore the anti-implantation effect of MP can be of diverse nature. They are closely interrelated rather than independent events, but the relations between one another are still not clear. Possibly, prevention of pregnancy after MP treatment on PD_{1-2} may be solely due to a 'tube locking' of eggs in the oviduct. Ova developing asynchronously with the uterus will degenerate if the endometrium is 'older' than the egg (Noyes & Dickmann, 1960). Furthermore, at the time of implantation, inhibition of decidual formation may play a role in the termination

of pregnancy. A significant difference in the serum concentration of E_2 and Δ^4P is observed between controls and MP treated rats. To what extent this altered serum hormone level induced by MP affects the oviductal transport and uterine sensitization awaits further investigations. In order to elucidate the whole action spectrum of MP on ovum implantation, studies on the rate of ovum transport and blastotoxic effect by the ovum transfer technique seem inevitable. Moreover, histological studies of the uterus during the period of implantation will clarify the effect of MP on the orientation of blastocyst and on uterine preparation.

Apart from these considerations, investigation using isolated components of the drug will definitely give more meaningful answers. Since crude extract of MP is used in the present experiments, it is indispensable to determine whether there is one or more active principle(s) involved and whether it is these compounds or their metabolites that brought about the physiological responses in the treated animals. Isolation of the active component(s) is now in progress.

5 SUMMARY

1. The effects of *Murraya paniculata* extracts on implantation were investigated in the rat. Oral administration of MP-EtOH in a minimum of 15 gm DWE was effective in suppressing implantation. The effective dosage of the chloroform and butanol fractions were 20 and 60 gm DWE, respectively.
2. MP was most effective when given on PD_{1-2} and was not active on PD_3 or thereafter.
3. Ovum implantation was delayed for approximately 24 hrs in MP treated rat.
4. Like the effect of estrogen, MP produced a sustained increase in uterine weight in intact and ovariectomized immature rat. It stimulated vaginal cornification in both pseudopregnant and ovariectomized rat.
5. MP produced a long term retention of nuclear estrogen receptor parallelled with a full replenishment of cytoplasmic receptor.
6. MP, in a dosage of 10 gm DWE, was effective in inhibiting decidual formation.
7. Concentrations of E_2 in serum were significantly reduced in MP treated rat on PD_3 and PD_5. On the other hand, serum concentrations of Δ^4P were significantly increase in MP treated rat on PD_5.

8. A single injection of E_2 on PD_2 or PD_4 failed to abolish the anti-implantation effect of MP.
9. MP exhibited no adverse effect on the development of the fetuses.
10. Normal mating and pregnancy occurred after the pseudopregnancy consequent after MP administration.

6 REFERENCES

Anderson, J.N., Clark, J.H. and Peck, E.J. Jr. (1972) Estrogen and nuclear binding sites : determination of specific sites by ^{3}H-estradiol exchange, Biochem. J. 126:561

Anderson, J.N., Peck, E.J. Jr. and Clark, J.H. (1975) Estrogen-induced uterine responses and growth : relationship to receptor estrogen binding by uterine nuclei, Endocrinology 96:160

Anon (1970) Zhong Cao Yao Tong Xun (5,6) :54 through Encyclopedia of Chinese Medicine, pp. 45, People's Press, People's Republic of China, 1977

Anon (1971) Guang Zhou Xin Yi Yao Tong Xun (3):46 through Encyclopedia of Chinese Medicine, pp. 45

Anon (1973) Clinical report of the ecobolic effect of *M. paniculata*, Fu Jian Sheng Yi Yao Yan Jin Suo through Encyclopedia of Chinese medicine, pp. 47

Anon (1977) Encyclopedia of Chinese Medicine, pp. 47, People's Press, Shanghai, People's Republic of China

Armstrong, D.T., Miller, L.S. and Knudsen, K.A. (1969) Regulation of lipid metabolism and progesterone production in rat corpora lutea and ovarian interstitial elements by prolactin and luteinizing hormone, Endocrinology 85:393

Armstrong, D.T., O'Brien, I. and Greep, R.O. (1964) Effects of luteinizing hormone on progestin biosynthesis in the luteinized rat ovary, Endocrinology 75:488

Bahl, O.P., Pandian, M.R. and Ghai, R.D. (1976) Immunological properties of the β-subunit of human chorionic gonadotrophin, Biochem. Biophys.Res. Comm. 70:525

Banik, U.K. and Pincus G. (1964) Estrogens and transport of ova in the rat, Proc. Soc. Exp. Biol. Med. 116:1032

Bartsch, W., Sponer, G., Dietmann K. and Fuchs, G. (1976) Acute toxicity of various solvents in the mouse and rat, Arzneimittel-Forsch. 26:1581-1583, through WHO Special Programme in Human Reproduction CP210: Preparation of suitable dosage forms of plant extracts for pharmacological evaluation in animal

Behrman, H.R. and Armstrong, D.T. (1969) Cholesterol esterase stimulation by luteinizing hormone in luteinized rat ovaries, Endocrinology 85: 474

Bennett, J.P. (1975) Drug regulation of egg transport, in Ovum Transport and Fertility Regulation pp.441 (Harper, M.J.K.; Pauerstein, C.J., Adam, C.E., Coutinho, E.M., Croxatto, H.B. and Paton, D.M. eds.) Bogtrykkeriet Forum, Copenhagen

Bennett J.P. and Kendle, K.E. (1967) The effect of reserpine upon the rate of egg transport in the fallopian tube of the mouse. J. Reprod. Fertil. 13:345

Berezovskaya, I.V. and Rudzit E.A. (1976) Effect of various solvents on the acute toxicity of drugs. Khim-Farm. Zh. 10(12):39-41 through Chem. Abstr. 86:100912t

Bindon, B.M. (1969) The role of the pituitary gland in implantation : inhibition of implantation by hypophysectomy and neurodepressive drugs. J. Endocrinol. 43:225

Callantine, M.R., Humphrey, R.R., Lee, S.L., Windsor, B.L., Schottin, N.H. and O'Brian, O.P. (1966) Action of an estrogen antagonist on reproductive mechanisms in the rat. Endocrinology 79:153

Chakraborty, D.P., Bhattacharyya, P., Roy S., Bhattacharyya, S.P. and Biswas, A.K. (1978) Strucrure and synthesis of mukonine, a new carbazole alkaloid from *Murraya koenigii*. Phytochemistry 17(4):834

Chang, M.C. and Harper, M.J.K. (1966) Effects of ethinyl estradiol on egg transport and development in the rabbit. Endocrinology 78:860

Chang, M.C. and Yanagimachi R.Y. (1965) Effect of estrogens and other compounds as oral antifertility agents on the development of rabbit ova and hamster embryos. Fertil. Steril. 16:281

Cheung, S.C. (1970) Ling Nan Cai Yao Lu (Revised 3rd ed.) pp. 188. The Institute of Present-day Chinese Medicine eds.

Chopra, N., Nayar, S.L. and Chopra, I.C. (1956) Glossary of Indian Medicinal Plants, pp. 171, C.S.I.R., New Delhi.

Clark, J.H., Anderson, J.N., and Peck, E.J. Jr. (1972) Receptor-estrogen complex in the nuclear fraction of rat uterine cells during the estrous cycle. Science 176:528

Clark, J.H.., Anderson, J.N. and Peck, E,J, Jr. (1973) Estrogen receptor antiestrogen complex : Atypical binding by uterine nuclei and effects on uterine growth. Steroids 22:707

Clark, J.H., Anderson, J.N. and Peck, E.J. Jr. (1974) Oestrogen receptors and antagonism of steroid hormone action. Nature 251:446

Clark, J.H., Paszko, Z. and Peck, E.J. Jr. (1977) Nuclear binding and retention of the receptor estrogen complex : Relation to the agonistic and antagonistic properties of estriol. Endocrinology 100:91

Cochrane, R.L. and Meyer, R.K. (1957) Delayed nidation in the rat induced by progesterone. Proc. Soc. Exp. Biol. 96:155

Corner, G.W. (1928) Physiology of the corpus luteum : The effect of very early ablation of the corpus luteum upon embryos and uterus. Am. J. Physiol. 86:74

Coutinho, E.M., Maia, H.S. and Adeodata-Filko, J. (1970) Responses of the human fallopian tube to adrenrgic stimulation. Fertil. Steril. 21:590

Croxatto, H.B., Diaz, S., Fuentealba, B., Croxatto H.D., carrielo, D. and Fabres, C. (1972) Studies on the duration of egg transport in the human oviduct. I. The time interval between ovulation and egg recovery from the uterus in normal women. Fertil. Steril. 23:447

De Feo, V.J. (1963a) Temporal aspect of uterine sensitivity in the pseudopregnant or pregnant rat. Endocrinology 72:305

De Feo, V.J. (1963b) Determination of the sensitive period for the induction of deciduomata in the rat by different inducing procedures. Endocrinology 73:488

De Feo, V.J. (1967) Decidualization. In : Cellular Biology of The Uterus (Wynn, R.M. ed) pp. 191 Applaton-Century-Crofts, New York.

Doehler, K.D. and Wuttke, W. (1974) Total blockade of phasic pituitary prolactin release in rats : Effect of serum LH and progesterone during the estrous cycle and pregnancy. Endocrinology 94:1595

Dreisbach, R.H. (1959) The Effect of steroid sex hormones on pregnant rats. J. Endocrinol. 18:271

Edgren, R.A. and Shipley, G.C. (1961) A quantitative study of the termination of pregnancy in rats with estrone. Fertil. Steril. 12:178

Ellinger J.V. and Kirton, K.T. (1972) Ovum Transport in rabbits injected with prostaglandin E_1 or $F_{2\alpha}$. Biol. Reprod. 7:106 (Abstr.)

Emmens, C.W. (1965a) The estrogenic and antifertility activities of diethylstilbestrol and dimethylstilbestrol in rodents. Acta Endocrinol. (Kbh.) 49:83

Emmens, C.W. (1953b) Estrogenic, antiestrogenic and antifertility activities of various compounds. J. Reprod. Fertil. 9:277

Emmens, C.W. and Martin, L. (1965) Biological activities of U-11100A. J. Reprod. Fertil. 9:269

Emmens, C.W., Cox, R.I. and Martin, L. (1964) The estrogenic and antiestrogenic activity of compounds related to diethylstilbestrol. Acta Endocrinol. (Kbh.) Suppl. 90:61

Fauvel, M.T., Gleye, J., Moulis, C. and Fouraste, I. (1978) Alcaloiedes De *Murraya paniculata* (L) Jack. Plantes Medicinales et Phytotherapie tome XII, n° 3, 207

Finn, C.A. (1965) Estrogen and the decidual cell reaction of implantation in mice. J. Endocrinol. 32:223

Finn, C.A. (1966) Endocrine control of endometrial sensitivity during the induction of the decidual cell reaction in the mouse. J. Endocrinol. 36:239

Finn, C.A. (1971) Biology of decidual cells. Adv. Reprod. Physiol. 5:1

Finn, C.A. and Emmens, C.W. (1961) The effect of dimethylstilbestrol and estradiol on deciduoma formation in the rat. J. Reprod. Fertil. 2:528

Ferrando, G. and Nalbandov, A.V. (1968) Relative importance of histamine and estrogen on implantation in rats. Endocrinology 83:933

Finn, C.A. and Keen, P.M. (1963) The induction of deciduomata in the rat. J. Embryol. Exp. Morph. 11:673

Finn, C.A. and Martin, L. (1970) The role of estrogen secreted before estrus in the preparation of the uterus for implantation in the mouse. J. Endocrinol. 47:431

Fuchs, A.R. and Mok, E. (1974) Histochemical study of the effects of prostaglandin $F_{2\alpha}$ and E_2 on the corpus luteum of pregnant rats. Biol. Reprod. 10:24

Ganguly, S.N. and Sarkar, A, (1970) Exozoline, a new carbazole alkaloid from the leave of *M. exotica*. Phytochemistry 17:1816

Ganguly, S.N.;Ghosh, S. and Basak, A. (1977) Coumarin from *M. paniculata*. Trans. Bose Res. Inst. 40(4):123

Gore, B.Z.; Caldwell, B.V; and Speroff, L. (1973) Estrogen-induced human luteolysis. J. Chin. Endocrinol. Metab. 36:615

Gorski, J.; Toft, D.; Shyamela, G; Smith, D.; and Notides, A. (1968) Hormone receptors : Studies on the interaction of estrogen with uterus. Recent Progr. Horm. Res. 24:45

Gray, A.I. and Waterman, P.G. (1978) Coumarins in the Rutaceae (Review). Phytochemistry 17:845

Greenwald, G.S. (1961) The antifertility effects in pregnant rats of a single injection of estradiol cyclopentyl-propionate. Endocrinology 69:1068

Greenwald, G.S. (1965) Effects of a dihydronaphthalene derivative (U-11100A) on pregnancy in the rat. Fertil. Steril. 16 : 185

Greenwald, G.S. (1967) Species differences in egg transport in response to exogenous estrogen. Anat. Rec. 157 : 163

Gupta, G.L. and Nigam, S.S. (1971) Chemical examination of the leaves of *M. koenigii*. Planta Med. 19(1):83

Hafez, E.S.E. (1975) Anatomical and physiological mechanisms of ovum transport. In Ovum Transport and Fertility Regulation. (Harper, M.J.K.; Pauerstein, C.J.; Adams, C.E.; Coutinho, E.M.; Croxatto, H.B.; Paton, D.M. eds.) pp. 361 Dogtrykkeriet Forum, Copenhagen.

Hamilton, T.H. (1968) Control by estrogen of genetic transcription and translation. Science 161:649

Hanson, F.W.; Powell, J.E. and Stevens, V.C. (1971) Effects of HCG and human pituitary LH on steroid secretion and functional life span of the human corpus luteum. J. Clin. Endocr. Metab. 32:211

Harper, M.J.K. (1967a) Effects of androstenedione on pre-implantation stages of pregnancy in rats. Endocrinology 81:1091.

Harper, M.J.K. (1967b) Effects of androstenedione on early pregnancy in the hamster. Endocriology 80:1152.

Harper, M.J.K. (1968a) Interaction with early pregnancy in rats by estrogen : Mechanism of action of dienestrol. Anat. Rec. 162:433

Harper, M.J.K. (1968b) Pharmacological control of reproduction in women. Prog. Drug Res. 12:47.

Harper, M.J.K. (1968b) Agents with antifertility effects during pre-implantation stages of pregnancy. In Biology of Mammalian Fertilization and Implantation, pp. 431 (Moghissi, K.S. and Hafez, E.S.E. eds) Springfield, Charles C Thomas Press.

Harper, M.J.K.; Bennett, J.P.; Boursnell, J.C. and Rowson, L.E.A. (1960) An autoradiographic method for the study of egg transport in the rabbit fallopian tube. J. Reprod. Fertil. 1:249

Harrington, E.F. (1965) Transportation of ova and zygotes through the genital tract of immature mice treated with gonadotrophin. Endocrinology 77: 635

Hashimoto, I, and Weist, W.G, (1969) Luteotrophic and luteolytic mechanisms in rat corpora lutea. Endocrinology 84:886

Hilliard, J.; Hayward, J.N. and Sawyer, C.H. (1964) Postcoital patterns of secretion of pituitary gonadotrophin and ovarian progestin in the rabbit. Endocrinology 75:957

Hilliard, J., Spies, H.G.; Lucas, L. and Sawyer, C.H, (1968) Effect of prolactin on progestine release and cholesterol storage by rabbit ovarian interstitium. Endocrinology 82:122

Hodgen, G.D.; Dufan, M.L. and Tullner, W.W. (1972) Estrogens, progesterone and chorionic gonadotropin in pregnant rhesus monkeys. Endocrinology 91:896

Huang,W.Y. and Pearlman, W.H. (1962) The corpus luteum and steroid hormone formation. I. Studies on luteinized rat ovarian tissue *in vitro*. J. Biol. Chem. 237:1060

Humphrey, K.W. (1968) The effects of estradiol-3-17β on tubal transport in the laboratory mouse. J. Endocrinol. 42:17

Humphrey, K.W. (1969) Induction of implantation of blastocysts transferred to ovariectomized mice. J. Endocrinol. 44:299

Humphrey, K.W. and Martin, L. (1968) The effect of estrogen and anti-estrogens on ovum transport in mice. J. Reprod. Fertil. 15:191

Jaffe, R.B.; Lee, P.A. and Midglay, A.R. (1969) Serum gonadotrophins before at the inception of, and following human pregnancy. J. Clin. Endocr. Metab. 29:1281

Jensen, E.V. and DeSombre, E.R. (1972) Mechamism of action of the female sex hormones. Ann. Rev. Biochem. 41:203

Kan, W.S. (1972) Manual of Medicinal Plants in Taiwan 2:377 National Research Institute of Chinese Medicine ed. Taiwan, Republic of China.

Katzenellenbogen, B.S. (1975) Synthesis and inducibility of the uterine estrogen-induced protein, IP, during the rat estrous cycle : Clues to uterine estrogen sensitivity. Endoctinology 96:289

Katzenellenbogen, B.S. and Gorski, J. (1972) Estrogen action *in vitro* : Induction of the synthesis of a specific uterine protein. J. Biol. Chem. 247:1299

Katzenellenbogen, B.S. and Ferguson, E.R. (1975) Antiestrogen action in the uterus : Biological ineffectiveness of nuclear bound estradiol after antiestrogen. Endocrinology 97:1

Katzenellenbogen, J.A.; Johnson, H.J. Jr. and Carlson, K.E. (1973) Studies on the uterine, cytoplasmic estrogen binding protein. Thermal stability and ligand dissociation rate. An assay of empty and filled sites by exchange. Biochemistry 2(21):4092

Komboj, V.P.; Chandra, H.; Satty, B.S. and Kar, A.B. (1970) Biological properties of 2-phenyl-3-p-(beta-pyrrolidnoethoxy)-phenyl-(2:1,b) naphthofuran - A new oral antifertility agent. Contraecption 1:29

Krehbiel, R.H. (1941) The effects of theelin on delayed implantation in the pregnant lactating rat. Anat. Rec. 81:381

Lau, I.F. Saksena, S.K. and Chang, M.C. (1973) Anti-implantation effect of Indomethacin in mice. Prostaglandins 4:795

Loeb, L. (1908) The experimental production of the maternal part of the placenta in the rabbit. Proc. Soc. Exp. Biol. Med. 5:102

Loewit, K.and Lawrence, K. (1970) Termination of pregnancy in the rat with rabbit antibovine LH serum. Fert. Steril. 20:679

Madhwa Raj, H.G. and Moudgel, N.R. (1970) Hormonal control of gestation in the intact rat. Endocrinology 86:874

Madhawa Raj, H.J.; Sairman, M.R. and Moudgal, N.R. (1968) Involvement of luteinizing hormone in the implantation process of the rat. J. Reprod. Fertil. 17:335

Mantle, P.G. (1969) Interruption of early pregnancy in mice by oral administration of agroclavine and sclerotia of *Claviceps fusiformis* (Loveless). J. Reprod. Fertil. 18:81

Mantle, P.G. and Finn, C.A. (1971) Investigations on the mode of action of D-6-methyl-8-cyanomethylergoline in supressing pregnancy in the mouse. J. Reprod. Fertil. 24:441

Marsh, J.M.; Telegdy, G. and Savard, K. (1966) Effect of gonadotropins on steroidogenesis in rat ovaries at dioestrus. Nature 212:950

Martin, L.; Cox, R.I. and Emmens, C.W. (1963) Further studies on the effects of estrogens and antiestrogens on early pregnancy in mice. J. Reprod. Fertil. 5:239

Mastroianni, L. Jr.; Abdul-Karim, R., Shah, U. and Segal, S.J. (1961) Changes in the secretion rate of the rabbit oviduct following oral administration of 1-(p-2-dimethylaminoethoxy-phenyl)-1-phenyl-2-p-anisyl-ethanol. Endocrinology 69:396

McCormack, J.T. and Greenwald, G.S. (1974) Evidence for a pre-implantation rise in estradiol-17β levels on day 4 of pregnancy in the mouse. J. Reprod. Fertil. 41:297

McDonald, G.J.; Armstrong,D.T. and Greep, R.O. (1967) Initiation of blastocyst implantation by luteinizing hormone. Endocrinology 80:172

McLure, T.J. (1966) Infertility in mice caused by fasting at about the time of mating. 1. Mating behaviour and littering rates. J. Reprod. Fertil. 12:243

Mester, I. (1973) The occurrence of alkaloids in Rutacea. Fitoterapia (Milano) 44:123

Meater, I. (1977) The occurrence of alkaloids in Rutacea. Addendum I. Fitoterapia (Milano) 48:268

Milgrom, E.; Atger, M. and Baulieu, E-E. (1970) Progesterone in uterus and plasma. IV. Progesterone receptors in guinea pig uterus cytosol. Steroids 16:741

Moon, Y.S.; Dorrington, J.H. and Armstrong, D.T. (1975) Stimulatory action of follicle-stimulating hormone on estradiol-17β secretion by hypophysectomized rat ovaries in organ culture. Endocrinology 97:244

Morishiga, W.K.,; Pepa, G.J. and Rothchild, I. (1973) The luteinizing hormone, prolactin and progesterone levels during pregnancy in the rat. Endocrinology 92:1527

Morris, J.M. (1970) Postcoital antifertility agent and their teratogenic effect. Contraception 2(2):85

Morris, J.M.; van Wagenen, G. and Dorfman, R.I. (1971) The antifertility activity of DL-cis-bisdehydrodisynolic acid methyl ether. Contraception 4:15

Morris, J.M. and van wagenen, G. (1973) Interception : The use of postovulatory estrogens to prevent implantation Am. J. Obstet. Gynecol. 115:101

Murr, S.M.; Bradford, G.E. and Geschwind, I.I. (1974) Plasma luteinizing hormone, follicle-stimulating hormone and prolactin during pregnancy in the mouse. Endocrinology 94:112

Niswender, G.D.; Menon, K.M.J. and Jaffe, R.B. (1972) Regulation of the corpus luteum during the menstrual cycle and early pregnancy. Fertil. Steril. 23:432

Noyes, R.W. and Dickmann, Z. (1960) Relationship of ovular age to endometrial development. J. Reprod. Fert. 1:186

Nutting, E.F. and Meyer, R.K. (1963) Implantation delay, nidation and embryonal survival in rats treated with ovarian hormones. In Delayed Implantation, pp 233. (Enders, A.C. ed.) University of Chicago Press.

O,Malley, B.W. and Means, A.R. (1974) Female steroid hormones and target cell nuclei. Science 183:610

Oriol-Bosch, A. and Cortes, J. (1975) Effects of post-ovulatory estradiol benzoate administration on women's ovarian function. Fertil. Steril. 26:405

Parkes, A.S. and Bellerby, C.W. (1926) Studies on the internal secretions of the ovary. II. The effects of an injection of the oestrus-producing hormone during pregnancy. J. Physiol. 62:145

Pauerstein, C.J.; Fremming, B.D.; Hodgson, B.J. and Martin, J.E. (1973) The promise of pharmacological modification of ovum transport in contraceptive development. Amer. J. Obstet. Gynec. 116:161

Pincus, G.;Banik, U.K. and Jacques, J. (1964) Further studies on implantation inhibitors.Steroids 4:657

Porter, D.G. and Finn, C.A. (1977) The biology of the uterus. In Frontiers in Reproduction and Fertility Control : A Review of The Reproductive Sciences and Contraceptive Development, Part 2, pp. 146 (Greep, R.O. and Koblinsky, M.A. eds.) MIT Press

Psychoyos, A. (1963) A study on the hormonal requirements for ovum implantation in the rat by means of delayed-nidation inducing substances. J. Endocrinol. 27:337

Psychoyos, A. (1971) Methods for studying changes in capillary permeability of the rat endometrium. In Methods in Mammalian Embryology, pp. 334. (Denial, J. C. and Freeman, Jr. eds.) San Francisco.

Raud, H.R. (1974) The regulation of ovum implantation in the rat by endogenous and exogenous FSH and prolactin; possible role of ovarian follicles. Biol. Reprod. 10:327

Revesz, C.; Chappel. C.L. and Goudry, R. (1960) Masculinization of female fetuses in the rat by progestational compounds. Endocrinology 66:140

Rochefort, H.; Lignon, F. and Capony,F. (1972) Formation of estrogen nuclear receptor in uterus : effect of androgens, estrone, and nafoxidine. Biochem. Biophys. Res. Commun. 47:662

Rothchild, I. and Meyer, R.K. (1942) Studies of the pre-trauma factors necessary for placentoma formation in the rat. Physiol. Zooel. 15:216

Rothchild, I.;Meyer, R.K. and Spielman, M.A. (1940) A quantitative study of estrogen-progesterone interaction in the formation of placentomata in the castrate rat. Am. J. Physiol. 128:213

Ruh, T.S.; Katzenellenboben, B.S., Katzenellbogen, J.A. and Gorski, J. (1973) Estrone interaction with the rat uterus : *In vitro* response and nuclear uptake. Endocrinology 92:125

Saksena, S.K.; Lau, I.F. and Chang, M.C. (1976) The inhibition of the conversion of testosterone into 5-alpha-dihydrotestosterone in the reproductive organs of the male rats. Steroids 27:751

Saksena, S.K.; Lau, I.F. and Chang, M.C. (1977) Determination of seven unconjugated steroids in the blood and seminal plasma of the fertile male rabbit. Hormone Research 8:117

Saksena, S.K.; Lau, I.F. and Chang, M.C. (1978) Estrogens in the seminal vesicle fluid and the effect of exogenous estradiol-17β on hormonal profile in the male rat. Biol. Reprod. 18:521

Sanyal, P.K.; Basak, A.; Barua, A.K. and Bose, P.K. (1975) Chemical Examination of the leaves of *Murraya paniculata*. J. Indian Chem. Soc. 52(12):1213

Saxena, B.B.; Hasan, S.H.; Haour, F. and Schmidt-Golluritzer, M. (1974) Radioreceptor assay of human chorionic gonadotrophin : detection of early pregnancy. Science 184:793

Segal, S.J. (1977) Systemic contragestational agents. In Frontiers in Reproduction and Fertility Control : A Review of the Reproductive Sciences and Contraceptive Development. Part 2, pp. 170 (Greep, R.O. and Koblinsky, M.A.) MIT Press.

Segal, S.J. and Nelson, W.O. (1958) An orally active compound with antifertility effects in rats. Proc. Soc. Exp. Biol. Med. 98:431

Selye, H. and McKeown, T. (1935) Studies on the physiology of the maternal placenta of the rat. Proc. R. Soc. B. 119:1

Shaikh, A.A. (1971) Estrone and estradiol levels in the ovarian venous blood from rats during the estrus cycle and pregnancy. Biol. Reprod. 5: 297

Shaikh, A.A. and Abraham, G.E. (1969) Measurement of estrogen surge during pseudopregnancy in rats by radioimmuno-assay. Biol. Reprod. 1:378

Shelesnyak, M.C.; Kraicer, P.F. and Zeilmaker, G.H. (1963) Mechanism of nidation.I. The estrogen surge of pseudo-pregnancy and progravidity and its role in the process of decidualization. Acta Endocrinol. 42:225

Smith, D.M. (1968) The effect of implantation of treating cultured mouse blastocysts with estrogen *in vitro* and the uptake of (^{3}H) estradiol by blastocysts. J. Endocrinol. 41(1):17

Smith, D.M. and Biggers, J.D. (1968) The estrogen requirement for implantation and the effect of its dose on the implantation response in the mouse. J. Endocrinol. 41:1.

Smith, M.S.; Freeman, M.E. and Neill, J.D. (1975) The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat : prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96:219

Spona, J.; Matt, K. and Schneider, W.H.F. (1972) Study on the action of D-norgestrel as a postcoital contraceptive agent. Contraception 11:31

Staples, R.E. (1966) Effect of clomiphene on blastocyst nidation in the rat. Endocrinology 78:82

Thiersch, J.B. (1962) Effects of substituted mecaptopurines on the rat litter in uterus.J. Reprod. Fertil. 4:291

Thomas, P.J. (1973) Steroid hormones and their receptors. J. Endocrinol. 57:333

Vande Wiele, R.L.; Bogumil, J.; Dyrenfurth, I.; Ferin, M. Jewelewicz, R.;Warren, M.; Rizkallah, T. and Khail, G.M. (1970) Mechanisms regulating the menstrual cycle in women. Recent. Preg. Horm. Res. 26:63

Velarado, J.T. and Hisaw, F.L. (1951) Quantitative inhibition of progesterone by estrogens in development of deciduoma. Endocrinology 49:530

Velarado, J.T.; Dawson, A.B.; Olsen, A.G. and Hisaw, F.L. (1953) Sequence of histological changes in the uterus and vagina of the rat during prolongation of pseudo-pregnancy associated with the presence of deciduomata. American J. of Anatomy 93:273

Watson, J.; Anderson, F.B.; Alam, M.; O'Grady, J.E. and Heald, P.J. (1975) Plasma hormones and pituitary luteinizing hormone in the rat during the early stage of pregnancy and after postciotal treatment with tamoxifen (ICI 46474). J. Endocrinol. 65:7

Weichert, C.K. (1941) The effectiveness of estrogen in shortening delayed pregnancy in the rat. Anat. Rec. 81:106

Weichert, C.K. (1942) The experimental control of prolonged pregnancy in the lactating rat by means of estrogen. Anat. Rec. 83:1

Weitlauf, H.M. and Greenwald, G.S. (1968) Survival of blastocysts in the uteri of ovariectomized mice. J. Reprod. Fertil. 17:515

Wiese, W.D.; Kidwell, W.R. and Balogh, K. (1968) Progesterone catabolism in the rat ovary: A regulatory mechanism for progestational potency during pregnancy. Endocrinology 82:844

Wu, J.T. and Meyer, R.K. (1970) The effect of implantation of cultruing delayed rat blastocysts in medium containing 17β estradiol. Biol. Reprod. 3:201

Yoshinaga, K. (1961) Effect of local application of ovarian hormones on the delay in implantation in lactating rats. J. Reprod. Fert. 2:35

Yoshinaga, K. Hawkin, R.A. and Stocker, J.F. (1969) Estrogen secretion by the rat ovary *in vivo* during the estrous cycle and pregnancy. Endocrinology 85:103

Yoshinaga, K.; Rice, C.; Krenn, J. and Pilot, R.L. (1979) Effects of nicotine on early pregnancy in the rat. Biol. Reprod. 20:294

Zeilmaker, G.H. (1963) Experimental studies on the effects of ovariectomy on blastocyst implantation in the rat. Acta Endocrin. 44:355